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FUNCTIONAL PROTEOMIC ANALYSIS OF THE MYOCARDIAL PKCε SUBPROTEOME AND PKCε-AKT-eNOS SIGNALING MODULES DURING CARDIOPROTECTION

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Dissertation Director

DEDICATION

For my parents

and my dear husband Lizhi

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ABSTRACT

FUNCTIONAL PROTEOMIC ANALYSIS OF THE MYOCARDIAL PKCε SUBPROTEOME AND PKCε-AKT-eNOS SIGNALING MODULES DURING CARDIOPROTECTION

Jun Zhang

September, 2003

Ischemic heart disease is the leading cause of mortality and morbidity in Western societies. Thus, understanding the molecular mechanisms to reduce myocardial ischemia and limit infarction size are of great importance. Ample evidence has shown that protein kinase C epsilon (PKCε) play an essential role in the genesis of cardioprotection. In particular, our laboratory has shown that activation of PKCε in the heart is sufficient to significantly reduce myocardial infarction due to coronary artery occlusion. However, the molecular mechanism responsible for PKCε-induced cardioprotection remain unclear. Recently, functional proteomic analysis have demonstrated that PKCε forms signaling complexes with various of proteins, including structural proteins, signaling molecules, stress-activated proteins, transcriptional/translational factors, metabolism-related proteins, and PKC-binding domain containing proteins, and the coordination of these molecules contributes to cardioprotection. Despite this information which obtained from whole heart lysates, little is known regarding the cellular mechanisms that regulate the assembly of these protein complexes and the specific manner in which these molecules interact with each. Therefore, this dissertation is focused on the characterization of PKCε subproteome and signaling modules within, as well as their regulation during cardioprotection. PKCε-Akt-eNOS signaling module is chosen as my research paradigm because the individual molecule PKCε, Akt and eNOS has

been implicated in the prevention of cell death. In addition, both eNOS and Akt were found to exist in the PKCε complex in our initial studies, suggesting that functional coupling among these molecules may be a heretofore unrecognized protective signaling mechanism. Our data demonstrate that the cardiac PKCε signaling subproteome is comprised of various sizes of protein complexes, and the formation of these complexes and modules is regulated by the subcellular localization and extracellular stimulus. During cardioprotection, activation of PKCε enhances the PKCε-Akt-eNOS module formation, and thus contributes to the regulation of nitric oxide production and hence the manifestation of the cardioprotective phenotype. In conclusion, the present research successfully characterizes the myocardial PKCε subproteome and PKCε-Akt-eNOS signaling modules, which serves as an important step towards our complete understanding of the signaling mechanisms underlying PKCε-mediated cardioprotection and ultimately aids in the development of pharmacological agents to protect the heart.

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CHAPTER I

General Introduction And Overview

Proteome, subproteome, and module

Upon completion of the human genome project, we have entered the era of proteomics. Proteomics is the study of the proteome, which was first defined in 1995 as all proteins expressed by the genome of an organism or tissue (175). Despite the power of genomic technologies, and the information derived from a sequenced genome, it is clear that proteins are ultimate functional units within cells, and the coordination of them determines the biological phenotype. Therefore, mapping the human proteome will bridge the gap between genomics and systems physiology, and provide tremendous information for diagnostic and therapeutic applications.

The goal of proteomics is to globally map protein expression profiles and protein-protein interaction profiles within the whole organism (133, 134). However, the generation of proteome map faces great challenges. First, one gene does not encode a single protein. Indeed, the number of proteins in the entire human body is expected to be many times higher than that of genes (81, 96). Second, concomitant co- and post-translational modification events, such as phosphorylation, glycosylation, sulphonation, palmitoylation, hydroxylation, acetylation and myristolation et al., which play critical roles in cellular function, can further increase the complexity of protein products from a single reading frame. For example, it is estimated that 30% of all cellular proteins contain covalently bound phosphate (94), and phosphorylation events control the majority of the signal transduction pathways in eukaryotic cells. Third, in contrast to the static genome, the proteome is a dynamic entity that is constantly being modulated by biological and environmental stimuli (74, 86). Fourth, the architecture of a proteome is both cell and organ specific. Finally, although significant advances have been made in the development of practical

proteomic techniques, more high-throughput platforms need to be evolved to enhance the power of current approaches (95). Taken together, mapping the entire proteome is a long-term project due to its enormity and complexity.

Instead of characterizing every protein within the cell, the goal of *functional* proteomic analysis is to analyze the spatial and temporal properties of the specific sets of proteins, called subproteomes in a cell, tissue, or organism, which are related to the particular biological functions (73, 170, 173). Compared with the tranditional proteomic analysis, functional proteomic studies are more feasible and advantageous. Proteins are not separate islands that perform their functions entirely by themselves. Instead, mounting evidence supports the concept that a protein may assembly of multi-protein complexes with other proteins to serve as a means to accomplish complicated tasks of cellular function (3). Characterization of the specific subproteome provides information about the components of the protein complex, and its dynamic changes, including protein expression, modification, and subcellular localization, in response to different conditions. So far, several studies (6, 22, 66, 71, 87, 98, 99, 101, 113, 132, 155) have undertaken the characterization of different subproteomes, including those defined by the MAPK cascade, the NMDA receptor complexes, nucleoporin complexes, and the PDGF receptor signaling system. Within the subproteome, two or more proteins are associated to form a basic functional unit of signal transduction, called a module, which conducts different tasks at various subcellular compartments. The formation of many distinct modules contributes to the establishment specific biological phenotype (170, 172).

A technical platform has been developed in our lab for comprehensive analysis of functional subproteomes (Figure 1) (135, 170, 171). In order to characterize proteins that associate with a protein of interest, isolation and purification of protein complexes is the first step. After subcellular fractionation which is an effective way to reduce the complexity and enrich the low-abundance proteins, immunoprecipitation and recombinant protein-based affinity pull down assays are commonly used to isolate protein complexes. Protein complexes are then separated by one- or two-dimensional electrophoresis, or liquid chromatography, followed by mass spectrometry or Western blotting to identify components within each complex. Several techniques

aid the further study of identification proteins, such as confocal microscopy to confirm the subcellular localization, biochemical *in vitro* assay to test the protein-protein interaction, and protein assays to detect the protein expression profiles within the subproteome. Finally, determination of the functional role of individual protein within the subproteome can link proteomic data to a physiological phenotype. This step can be conducted by using cell culture or transgenic animal models, or treatment of activators or inhibitors to test the biological significance of the protein change in response to the changed environmental conditions.

Similarly, the cardiac proteome is proposed to compose of multiple integrated functional subproteomes, each of which is organized to facilitate a unique function. Moreover, specific modules are formed within subproteomes in response to different stimuli (145, 160, 172). Instead of attempting to simultaneously examine all proteins in cardiac cells, we focus our research on characterization myocardial subproteomes that are critically involved in the development of ischemic injury and cardioprotection, especially the subproteomes of protein kinases associated with cardioprotection (136, 142, 172). Three aspects are important to characterize a subproteome: the identification of the proteins that compose the subproteome are classified according to their biological functions; the molecular architecture of the subproteome, the manner in which the subproteome is altered in different subcellular locations; and the dynamic modulation of the subproteome that underlies the development of a specific phenotype. Delineation of multiple subproteomes based on their respective biological functions and contributions to phenotypes will eventually lead to full characterization of cardiac proteome.

The goal of this dissertation was to contribute to this understanding of cell signaling in two fundamental regards. First, as detailed in Chapter III, we wanted to examine the role of subcellular-dependent regulation of signal transduction by testing the basic hypothesis that the cell recruits different proteins to regions of the cell to perform specific tasks, and that multiprotein complexes are involved in this phenomenon. Second, as reported in Chapter IV, we targeted investigation of a specific signaling module within the cardiomyocyte to test a separate but equally central hypothesis, namely, that the cell organizes multiple molecules into signal transduction units (or modules) that are mobilized to accomplish signaling events and to regulate

phenotype. In this regard, the present dissertation advances our understanding of cell signaling in general on two critical levels that are interrelated to the common theme of PKCε-mediated cardioprotection.

Ischemic injury and cardioprotection

Ischemic heart disease is the leading cause of mortality and morbidity in Western societies (25). Thus, mechanisms to reduce myocardial ischemia and to prevent myocytes death are of great importance to improve patient survival. Due to the limited ability of the heart to regenerate cardiomyocytes, myocardial cell death is the most deleterious event in the heart. In the past, numerous attempts had been undertaken to prevent myocardial cell death and limit the infarct size after a coronary artery occlusion. An endogenous protective defense mechanism termed ischemic preconditioning (PC) was discovered in 1986 (128), and exerts a very powerful anti-infarct effect. Ischemic PC is a phenomenon whereby brief ischemic episodes activate an innate protective system in the heart to render the heart powerfully resistance to subsequent prolonged lethal ischemic insults. Over the past 16 years, a tremendous evidence has demonstrated that this protective phenomenon exists in virtually all mammalian models(11, 23). It is hoped that elucidation of the intracellular signaling mechanisms underlying the myocardial preconditioning may lead to delivery of effective therapeutic agents capable of reducing the myocardial infarction. Importantly, cardioprotection can also be engendered by the administration of pharmacological agents such as nitric oxide (NO) donors *in lieu of* ischemia (140, 179, 181). NO donor-induced cardioprotection may have potential clinical applications.

Mounting evidence has demonstrated that protein kinase C epsilon plays a critical role in the genesis of cardioprotecion (49, 75, 117, 143, 186). In particular, cardiac-specific activation of PKCε affords an infarct-sparing effect similar in efficacy to that induced by ischemic and pharmacological PC. However, the molecular and cellular mechanisms underling PKCε-induced cardioprotection are poorly understood.

Protein kinase C epsilon (PKCε)

The protein kinase C (PKC) family of serine-threonine kinases is composed of 12 different isoforms that are divided into three subgroups based on their regulation. The classical or conventional PKCs (cPKCs) include the α, βI, βII, γ isoforms, and are regulated by Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine (PS). The novel PKCs (nPKCs), which include the ε, δ, η, and θ isoforms, are regulated by DAG and PS; but are insensitive to Ca^{2+}. The atypical PKCs (aPKCs) include the ζ, ι, and λ isoforms and are regulated by PS alone. The μ isoform of PKC (also known as PKD), which is sensitive to both DAG and PS, has been placed into a separate class because of its unique structural characteristics (129) (Figure 2).

Molecular structure of PKCε : Protein kinase C ε (PKCε) was first cloned in 1987 and defined as a novel PKC isoform. Akin to other PKCs isoforms, PKCε consists of two primary functional domains: an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain (129) (Figure 2). Within the regulatory domain, there are two C1 regions and one C2 region. The C1 region contains C1A and C1B which form the DAG/phorbol esters binding site via the cysteine-rich Zn^{2+} finger motif. An autoinhibitory pseudosubstrate sequence precedes the C1 domain which can autoinhibit PKCε to form an inactive closed conformation by occupying the catalytic substrate-binding cavity. Unlike cPKCs, the C2 region of PKCε lacks the specific aspartate residues which contribute to the Ca^{2+} binding. Therefore, activation of PKCε is not dependent upon the concentration of Ca^{2+} ions. The catalytic domain has an ATP-binding site (C3) and a substrate-binding site (C4), both of which are essential for kinase activity. The hinge region between C2 and C3 domain can be easily cleaved to release the carboxyl terminal constitutively active enzyme (kinase domain) when PKCε is membrane-bound (Figure 3).

Regulation of PKCε: There are at least three conserved phosphorylation sites within PKCε that are necessary for the maturation of PKCε and its ability to respond to second messengers. The first, rate-limiting step is the phosphorylation of Thr566 in the activation loop (kinase core). Phosphoinositide-dependent kinase-1 (PDK-1) may be involved in this phosphorylation event that leads to the exposure of C-terminus of PKC to the following two phosphorylation modifications via intracellular autophosphorylation or catalysis by other kinases (33, 161). These two phosphorylation sites are Thr710 at the turn motif (164), and Ser729 in hydrophobic site in C-terminus. Phosphorylation of these three sites results in conformational changes of PKCε that not only lock PKCε into a more thermally stable, protease- and phosphatase-resistant stage, but also free the substrate-binding cavity for pseudosubstrate to form the closed conformation (Figure 3).

Mature but inactive PKCε localizes in the cytosolic fraction where it is maintained in the autoinhibited closed conformation. PKCε can be activated by several different second messengers, including, DAG, PIP_3 (phosphatidylinositol 3,4,5-triphosphate), and fatty acids that are produced by physiological stimuli such as PDGF (platelet-derived growth factor) and bradykinin (72, 127). After stimulation, PKCε binds to membrane-associated DAG via its C1 domain. This stimulates the translocation of PKCε from cytosolic fractions to membrane fractions where C2 region can interact with membrane phosphatidyl serine (PS) (129). This tethering of both C1 and C2 regions to membrane lipids provide the energy to release the pseudosubstrate motif from the catalytic domain and open the enzyme's conformation, resulting in maximal PKCε activation.

Subcellular distribution of PKCε: The subcellular distribution behavior of PKCε depends on (i) which second messenger is bound to C1 domain and (ii) which intracellular compartment the PKCε-specific binding partner, called RACK2 (also known as β'-COP), is localized. This latter molecule binds the C2 (-like) domain of PKCε and modulates its activity. Therefore, both the C1 domain and C2 domain of PKCε function as subcellular localization signals (112). The spatial-

and temporal- regulation of PKCε subcellular localization may provide the explanation for its substrate specificity in the signaling transduction.

Function of PKCε in non-cardiac cells: PKCε is expressed in many tissues and cells, with particular abundance in neuronal, hormonal, and immune cells. PKCε has been reported to play essential roles in a variety of cellular signaling processes (2), including proliferation, differentiation, gene expression, muscle contraction, mechanical force adaptation, metabolism, transport, exocytosis, endocytosis, and inflammation. PKCε deficient mice show macrophage dysfunction due to decreased activation of the transcription factor NF-κB, resulting in increased mortality after bacterial infection, and indicating the important protective role of PKCε in the immune system (32). In the neuronal system, PKCε induces neurite outgrowth during neuronal differentiation through its interaction with actin filaments (106). PKCε has also been implicated in the regulation of nociceptor function that participates in the perception of pain (46). Moreover, PKCε could promote cell survival and prevent apoptosis through suppression of the exitotoxic glutamate receptor in neuronal PC12 cells (167), and through dysregulation of the mitochondrial caspase pathway in lung cancer cells (46).

PKCε and cardioprotection: In cardiac cells, multiple investigators have found that activation and translocation of PKCε serves as a central signaling event in the development of cardiac protection (75, 117, 140-142). In the past several years, studies from our laboratory (140-142) have shown that cardiac-specific overexpression of PKCε in transgenic mice hearts can mimic protection against myocardial infarction by ischemic preconditioning (Figure 4). Studies based on the cardio-specific transgenic expression of the PKCε–selective translocation inhibitor (epsilonV1) or activator (ψepsilon RACK) peptides from other groups also clearly show the importance of PKCε in the cardiac protection against ischemic damage as well as during normal postnatal myocardial development (50). Furthermore, moderate ethanol consumption exerts beneficial cardioprotective effects via mechanisms that involve PKCε activation (188).

Although PKCε has an established role in the genesis of cardioprotection, the molecular and cellular mechanisms responsible for PKCε-mediated cardiac protection remain unclear.

Mounting evidence suggests that PKC-dependent protection involves a battery of kinases (tyrosine kinases, Akt, PI3-K, mitogen-activated protein kinase (MAPKs)) (145, 146, 166), stress-activated proteins (heat shock proteins (HSPs), nitric oxide synthases (NOSs), ion channels, and pro-survival transcription factors (NF-κB, HIF-1α). Recently, using functional proteomic approaches, our laboratory has demonstrated that PKCε forms signaling complexes with multiple proteins and that the coordinated action of these molecules confers protection against ischemic injury (12, 20, 21, 130). In other words, there is a cardiac signaling network centered around PKCε that is critical for cardioprotection.

AKT / Protein Kinase B

Akt, also called protein kinase B (PKB), is a serine/threonine kinase, which was first discovered in 1991 by two independent research groups. Akt was initially characterized as the human cellular homologue of the viral oncogene *v-akt* from the transforming retrovirus AKT8 (19, 38, 100). To date, three major isoforms of Akt/PKB have been identified, encoded by three distinct gene loci, termed Akt1/α, Akt2/β, and Akt3/γ. All three isoforms share a high degree of homology at the amino acid level (42, 103). Akt2 and Akt3 are approximate 82% identical with the Akt1 isoform. Akt3 lacks 23 amino acids at the C-terminal compared with others (110) (Figure 5). All three isoforms are ubiquitously expressed in mammals, although levels of expression vary among tissues. Akt1 is the predominant isoform in most tissues, such as brain, thymus, heart and lung (19, 38, 100). Akt2 is primarily expressed in the insulin-responsive tissues, such as skeletal muscle, heart, liver and kidney (7). Expression of Akt3 is more restricted, appearing high in the brain and testes, and lower in heart, spleen, lung and skeletal muscle. All tissues contain at least one form of Akt.

Molecular structure of Akt/PKB: Examination of the amino acid sequence of Akt/PKB revealed that all three isoforms contain a highly conserved structure. The N-terminal 100 amino acids posses a pleckstrin homology (PH) domain that mediates lipid-protein or/and protein-protein interactions. A short glycine-rich region links the PH domain to the catalytic domain. The central kinase catalytic domain has a conserved phosphorylation site Thr308 (Akt2 Thr309, Akt3 Thr302) within the activation loop. In C-terminal regulatory domain there is another conserved phosphorylation site Ser473 (Akt2 Ser474, Akt3 Ser472) (Figure 5).

Regulation of Akt/PKB: In the inactive form of Akt, the PH domain serves as an inhibitory structure that sterically blocks the catalytic site (similar to the antoinhibition of PKCε). Activation of Akt is initiated by the binding of PIP_3 to the PH domain of this enzyme. This action not only

releases autoinhibition through conformational changes that unmask the active site, but also recruits Akt to the plasma membrane where it can be further phosphorylated and fully activated. So far, phosphorylation of Thr308 in the kinase activation loop and Ser473 in the C-terminal hydrophobic region are the two most important posttranslational modifications. Mutation of Thr308 to nonphosphorylatable alanine abolishes Akt 's activation, indicating that phosphorylation of Th308 is essential for activation of Akt. In contrast, mutation of Ser473 only partially inhibits Akt activation, suggesting that Ser473 phosphorylation facilitates maximal Akt activation but is not a necessary step in Akt-dependent signaling (5, 138, 183).

Ser124 and Thr450, which are constitutively phosphorylated, were also predicted to be prerequisite for Akt responsiveness (18), although, mutation of these residues to alanine has no effect on the activity of Akt (7, 165). In addition, a recent report finds that Src family tyrosine kinase dependent phosphorylation of two tyrosine residues (Y315, and Y326) may contribute to the activation of Akt (35).

The cellular activation of Akt/PKB is dependent upon the interaction with membrane-bound second messager PIP_3. The generation of PIP_3 is catalyzed by PI-3K via the phosphorylation of phosphoinositides (PtdIns). Although PIP_3 does not activate Akt/PKB directly, it recruits Akt to the plasma membrane and alters the conformation of Akt to facilitate the subsequent phosphorylation modifications. The upstream kinase that phosphorylates Thr308 is a 67kDa ubiquitously expressed kinase called PDK-1 (5, 162, 163). The role of PDK-1 to activate Akt has been supported by studies from embryonic stem cells deficient in PDK-1 (177), biochemical inhibition of PDK-1 (13), and by studies employing anti-sense oligonucleotides against PDK-1 (59). Akt/PKB becomes a substrate for PDK-1 once its PH domain engages PIP_3 on the membrane. Interestingly, as indicated above, PDK-1 is also known to phosphorylate and activate PKC, though the mechanisms contributing to the specificity of PDK-1 for PKC, Akt, or other substrates, remain poorly defined.

The upstream kinase for Ser473 phosphorylation is unknown, and is tentatively named "PDK-2". Several candidate activities have been proposed, including PKCζ (34, 190), an

unnamed lipid-raft-associated kinase (84), or simply an autophosphorylation processes following the PDK-1 activity (1).

There are two postulated models for Akt activation. The first mechanism in response to PI3K induction and PIP_3 production, Akt moves from the cytosol to the plasma membrane, where Ser473 phosphorylation occurs through autophosphorylation or by PDK2. This region forms a docking site for PDK-1 to phosphorylate Thr308 and hence activate Akt (8). The second mechanism is that cytosolic Akt and PDK-1 are co-localized, but Akt is inactive due to constraints imposed by its PH domain. Growth factors stimulate PI3K, which draw both PDK-1 and Akt to the plasma membrane. The PH domain of Akt binds to PIP_3, unmasking the activation loop and allowing PDK-1 to phosphorylate Thr308. Subsequent elevation of Akt activity promotes autophosphorylation of Ser473 or by a third-party enzyme (165). Once Akt is activated, it targets specific proteins in the cytoplasm, plasma membrane and and nucleus (Figure 5).

The consensus motif for Akt phosphorylation is R-x-R-x-x-S/T-F/L (x represents any amino acid) (5). So far, several potential Akt substrates have been found, such as glycogen synthase kinase-3 (GSK3), IκB-α kinase α (IKKα), Raf, and Bad. Recently, Akt was found to regulate the level of nitric oxide (NO) production through direct phosphorylation and activation of the endothelial isoform of nitric oxide synthase (eNOS) (44, 63).

Subcellular distribution of Akt/PKB: The subcellular localization of Akt is tightly regulated. Two minutes after stimulation by IGF-1, Akt1 is translocated to the plasma membrane in a PH domain-dependent manner. Prolonged stimulation induces Akt translocated to the nucleus by an unknown mechanism (8), which is PH-domain-independent.

Function of Akt/PKB: Ample evidence shows that Akt is a key player in numerous physiological and pathophysiological processes, such as gene transcription, protein synthesis, glycogen synthesis, transcription control, cell growth, cell survival and angiogenesis (26, 28, 44, 62, 63, 67, 69, 121,182). In cardiomyocytes, Akt is activated by gp130 stimulation and has both a hypertrophic and an antiapoptotic effect (62, 131). Bell and colleagues reported that administration of bradykinin, as an adjunct to reperfusion, limits infarct size in the mouse heart via induction of increased activity of PI3kinase, Akt and eNOS (62). In myocardial gene transfer

studies, a constitutively active mutant of Akt reduces infarct size and apoptosis after ischemia-reperfusion injury (62). Using DNA microarrays, Cook et al (40) reported the cardiac gene expression profiles in mice with cardiac-specific overexpression of Akt. Studies from these Akt mice also indicate that Akt exerts a dual function *in vivo* on the myocardium by increasing both cardiomyocyte size and contractility (39). They found that chronic Akt activation resulted in the differential regulation of at least 40 genes in the heart that may contribute to the effects of Akt on cardiomyocyte survival, metabolism, and growth. Interestingly, studies from Zhou's group find that moderate alcohol consumption induces sustained cardiac protection is through a PKCε– and Akt-dependent signaling pathway (188).

Endothelial nitric oxide synthase (eNOS)

Nitric oxide (NO) is a potent messenger molecule that participates in many physiological processes such as vascular relaxation, neuronal communication, immunological modulation, and host defense (4). NO is produced from oxidizing L-arginine to generate L-citrulline which is catalyzed by a family of NO synthases (NOS). There are three distinct isoforms of NOS, referred to as endothelial NOS (eNOS, NOS-3), neuronal NOS (nNOS, NOS-1), and inducible NOS (iNOS, NOS-2). eNOS and nNOS are Ca^{2+}/calmodulin-dependent enzymes producing NO in short bursts and low concentrations (nM), whereas iNOS produces NO in high concentrations (μM) and is Ca^{2+}-indenpedent (9).

Molecular structure of eNOS: eNOS was first identified and isolated from bovine aortic endothelial cells (9, 10) in 1991. Since then, it has been found in many cells, such as platelets (148), smooth muscle cells (156), cardiac myocytes (14), bone cells, (4) and neurons (27, 45). According to the amino acid sequence, the primary structure of eNOS has two functional domains: reductase domain and oxygenase domain. The C-terminal reductase domain (10) contains NADPH, FAD, FMN, and calmodulin (CaM) binding sites and the N-terminal oxygenase domain has binding sites for tetrahydrobiopterin (BH4), heme, and L-arginine (14, 27). In particular, the BH4 domain is reported to be critical for eNOS activity (116, 169), as it is involved in dimer formation and electron transfer, as well as maintenance of the active site's structural integrity (Figure 6).

The quaternary structure of eNOS includes its dimerization and association with other proteins. The eNOS enzyme is the only fully functional in a dimeric form. Dimerization of eNOS starts with the binding of heme (108), and the binding of BH4, to stabilize the dimer (102, 116, 150). The reductase domain transfers electrons from NADPH via the FAD, FMN and CaM co-factors to the heme in the oxygenase domain. The oxygenase domain catalyzes L-arginine to L-

citrulline with BH4 binding to O_2. CaM binding increases the rate of electron transfer, by which increased intracellular Ca^{2+} concentration is required for this protein-protein interaction. Hence, the Ca^{2+}/CaM-dependence is one of the features of this particular NOS isoform (Figure 6).

Regulation of eNOS: The regulation of eNOS occurs at three levels: gene transcription, co/post-translational modification, and allosteric regulation of the enzyme itself.

Transcriptional/translational control alters eNOS protein expression levels via regulation of the eNOS promoter and altered stability of eNOS mRNA. There are numerous transcription factor binding sites on the eNOS promoter, including activator protein (AP)-1, nuclear factor (NF)-1, nuclear factor-κB (NF-κB). Multiple stimuli can affect the basal expression of eNOS. For example, hypoxia leads to the activation of AP-1 and induction of eNOS transcription (53, 89). eNOS levels in endothelial cells also can be efficiently regulated by changes in eNOS mRNA stability. Hypoxia and cytokines, such as TNF, can downregulate eNOS mRNA levels by reducing the half-life of eNOS mRNA from 48 h to as little as 3 h (122, 125, 185, 187).

There are several phosphorylation modifications within the eNOS protein. Ser1177 (human) in the reductase domain is the most important one. It is stimulated by the application of fluid shear stress (44, 64), estrogen (111), VEGF (107), insulin (58), or bradykinin (16) and results in increased NO production. The kinases involved in this process vary with the stimuli and include Akt (44, 51), PKA (123), and CaMKII (120). In addition, Thr495 within the CaM-binding is constitutively phosphorylated in endothelium cells, possibly via PKC, and is associated with decreased eNOS activity (43, 85, 120, 123). Moreover, other phosphorylation sites, such as Ser114 within the oxygenase domain, are thought to be modified by ERK1/2 (56) and are related to the dimerization of eNOS. Ser633 and some tyrosine residues can also be phosphorylated, but the functional significance of these post-translational modifications remains to be determined (44, 56, 57).

Lastly, the molecular chaperone Hsp90 serves as an allosteric modulator (65) for eNOS activity. Activation of vascular endothelial growth factor, histamine or fluid shear stress in human endothelial cell increases the interaction between eNOS and Hsp90, and increases eNOS activity by approximate 3-fold.

Subcellular localization of eNOS: eNOS is largely membrane-associated as a result of N-terminal myristoylation and palmitoylation modifications (30, 152, 157). When eNOS is located in the caveolae, flask-shaped invaginations of the plasma membrane that occupy up to 30% of cell surface, it is bound to caveolin. Caveolin is a caveolar resident coat protein that inhibits eNOS activity by interfering with Ca^{2+}/CaM binding and electron transfer to the heme subunits (9). In endothelial cells, eNOS binds to caveolin-1, whereas in cardiac myocytes, eNOS is associated with caveolin-3. An increase in intracellular Ca^{2+} causes the formation of Ca^{2+}/CaM complexes, which displaces caveolin resulting in the dissociation of eNOS. At this point, eNOS is now activated and remains so until intracellular Ca^{2+} levels decrease and the Ca^{2+}/CaM complex can no longer compete with caveolin. The specific intracellular compartments to which eNOS translocates remain unclear, but many include the nuclear membrane, the endoplasmic reticulum, or trans-golgi system (83).

Function of eNOS: The protective effects of NO generated by eNOS are varied. It regulates vascular tone by relaxation of vascular smooth muscle cells (vasodilation) (76). It inhibits leukocyte and platelet adhesion and aggregation to endothelium (100) and inhibits vascular smooth muscle cell proliferation (156).

eNOS is the major form of NOS in the heart and it is expressed in endothelial cells and cardiac myocytes (14, 105). The protective role of eNOS in the cardiovascular system has been widely studied. Myocardial overexpression of eNOS in mice greatly stimulates cardiac NO formation and protects the heart against ischemia/reperfusion injury (29). Moreover, gene transfer of eNOS to the heart reduces ischemia/reperfusion injury by inhibiting NF-κB activation, adhesion molecule expression, and the early infiltration of leukocytes (92). In contrast, eNOS knockout mice exhibit increased left ventricular contractility in response to β-adrenergic agonists compared with wide type mice, demonstrating a negative inotropic effect of eNOS-derived NO (15, 70, 79). eNOS is also reported to be involved in the development of ischemic preconditioning. Xuan's group report that in conscious rabbit hearts, ischemic PC elicits an immediate activation of Ca^{2+}-dependent NOS (most likely eNOS), indicating that eNOS serves as a trigger on day 1 to initiate the development of late PC 24 hours later (181).

PKCε-Akt-eNOS signaling modules

As detailed above, PKCε plays a critical role in the genesis of cardioprotection against myocardial ischemic injury (49, 140-143, 147). Specifically, recent studies in our laboratory showed that PKCε forms multimeric signaling complexes with at least 93 proteins, including structural proteins, stress-activated proteins, proteins associated with transcription and translation, proteins involved in cellular metabolism, and various kinases and phosphatases (52, 143). Furthermore, the assembly of these multiprotein PKCε complexes is dynamically modulated in the PKCε-transgenic mouse hearts, which serves as important evidence for the role of these complexes in regulating the cardioprotective phenotype.

Importantly, among the 93 known protein partners, Akt and eNOS are of great interest as they are well established to independently promote cell survival and prevent cell death in response to various forms of stress (16, 42, 60, 63, 104, 124, 182). Taken in the context of the foregoing introduction regarding the essential roles of these three molecules in the cardiovascular system, the finding that PKCε, Akt and eNOS may be signaling partners in the myocardium was a tantalizing piece of evidence suggesting functional coupling of these molecules. As a result, we chose to focus our studies to define the specific role of subcellular dependent regulation of the PKCε subproteome, and in particular, of PKCε-Akt-eNOS signaling modules within this subproteome.

Accordingly, my research has focused on the identification of PKCε subproteome, and the signaling modules within. We hypothesized that PKCε form distinct signaling modules at different subcellular compartments with different protein components in the mouse heart, and moreover, that the assembly and activation of signaling modules conduct different tasks which are essential to the genesis of cardioprotection (Figure 7). In the first part of my research, I concentrated on the identification of PKCε subproteome in different subcellular compartments,

and the regulation of these protein complexes during cardioprotection. In the second part of my study, I focused on the characterization of PKCε-Akt-eNOS signaling modules, which were chosen as an example to functionally validate a signaling module in cardioprotection.

Previous studies in non-cardiac cells have provided evidence for physical and functional interaction between PKC, Akt and eNOS. Akt can directly interact and phosphorylate eNOS on Ser1177 (63). Doornbos' (48) and Mao's (119) groups reported that Akt can be co-immunoprecipitated with PKCζ or PKCι, and that the PH domain of Akt is implicated as the binding site for PKCζ to Akt1 (110) and Akt3 (88) in COS-1 cells. Li and colleagues also find that PKCα overexpression stimulates Akt activity and suppresses apoptosis induced by interleukin 3 withdrawals in myeloid progenitor cells (114). In contrast, Wen (176) reported that in A549 and HEK293 cells, the phosphatidylinositol 3-kinase/Akt signaling pathway is negatively regulated by PKC. Similarly, PMA-induced apoptosis is accompanied by an inhibition of Akt activity (149). Moreover, PKC activation inhibited eNOS activity by attenuating eNOS phosphorylation on Ser1177 and increasing phosphorylation of Thr495 in cardiovascular endothelial cells (123, 124).

Despite this information, nothing is known regarding the formation and regulation of PKCε-Akt-eNOS signaling modules in myocytes, especially during cardioprotection. Therefore, comprehensive investigations need to be implemented to draw a clearer picture of the temporal and spatial relationship among PKCε, Akt and eNOS in the regulation of NO production and the genesis of cardioprotection. Successful characterization of myocardial PKCε-Akt-eNOS signaling modules would serve as a good model for the future research addressing the signaling mechanisms underlying PKCε-mediated cardioprotection, and of critical importance in the development of pharmacological agents for the treatment of myocardial ischemia and infarction in patients. This dissertation provided new information about the molecular architecture of PKCε-Akt-eNOS signaling module, the subcellular localization of these modules, and the dynamic modulation of these modules during the development of the cardioprotective phenotype.

Figure 1. Functional proteomic platform for isolation and characterization of multiprotein complexes. This is a hypothesis-driven research strategy to characterize a cellular subproteome responsible for the genesis of a phenotype. Started with subcellular fractionation which is an effective way to reduce the complexity and enrich the low-abundance proteins, immunoprecipitation and recombinant protein-based affinity pull down assays are commonly used to isolate and purify protein complexes. Protein complexes are then separated by one- or two-dimensional electrophoresis, or liquid chromatography, followed by mass spectrometry or Western blotting to identify components within each complex. Then, biochemical analyses and phenotype assessment are conducted to determine the functional role of individual protein within the subproteome and link proteomic data to a physiological phenotype. We use this strategy to characterize the PKCε-dependent signaling mechanisms underlying cardioprotection.

Functional Proteomic Technology Platform

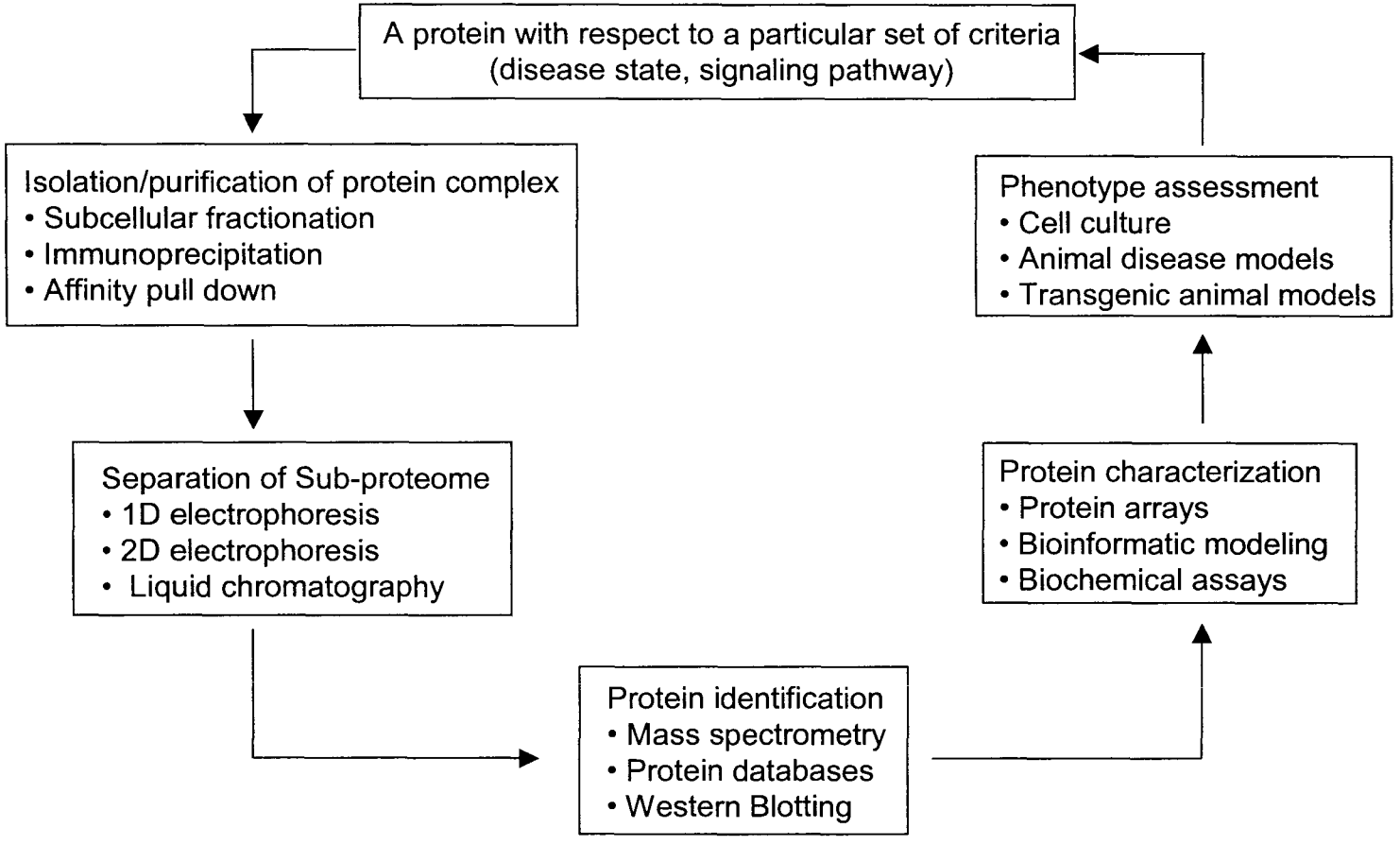
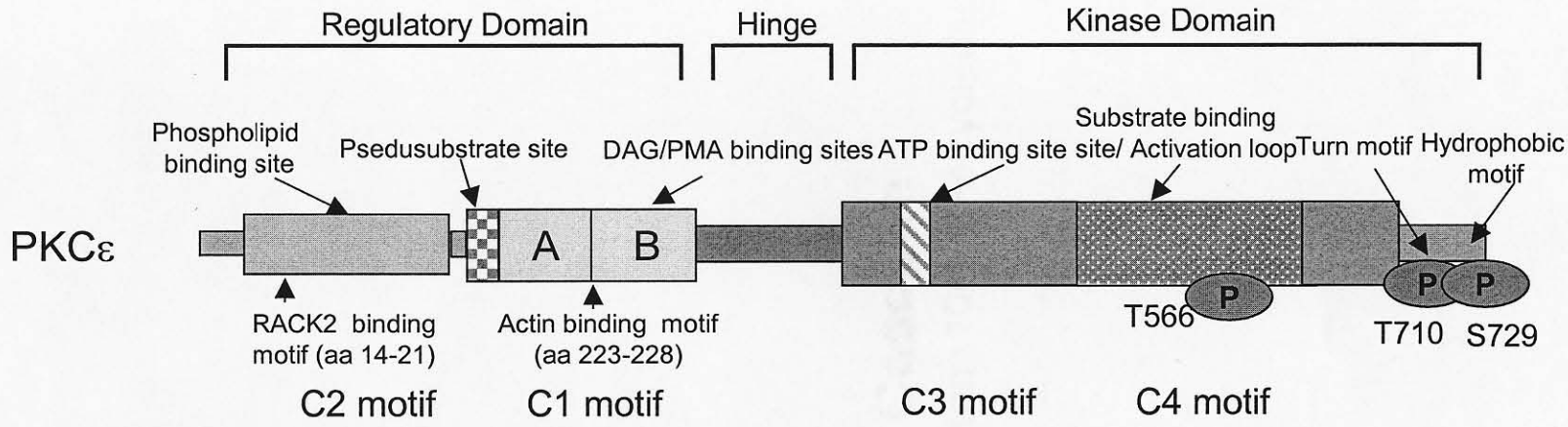


Figure 2. Molecular structure of protein kinase C epsilon (PKCε). Alike other PKCs, PKCε has regulatory domain and kinase domain. Within the regulatory domain, an autoinhibitory pseudosubstrate sequence is ahead of C1 motif (dotted red). 2nd messenger DAG and phorbol esters bind to C1 domain; whereas, acidic lipids (PS) binds to C2 domain. Kinase core has conserved three phosphorylation sites. N is the amino terminus. C is the carboxyl terminus.



Modified from Newton AC, *Biochem J*, 2003

Figure 3. Model of activation of PKCε. Newly synthesized immature PKCε need three conserved phosphorylation modifications to become maturation which is phosphorylated by PDK-1 followed by autophosphorylation. Mature but inactive PKCε localizes in cytosolic fraction. In response to various stimulus, the generation of DAG and PS recruits PKCε to the membrane fraction by engaging the C1 and C2 domains on the membrane. This membrane interaction provides the energy to release the pseudosubstrate motif from the substrate-binding cavity, resulting open confirmation and maximal PKCε activation.

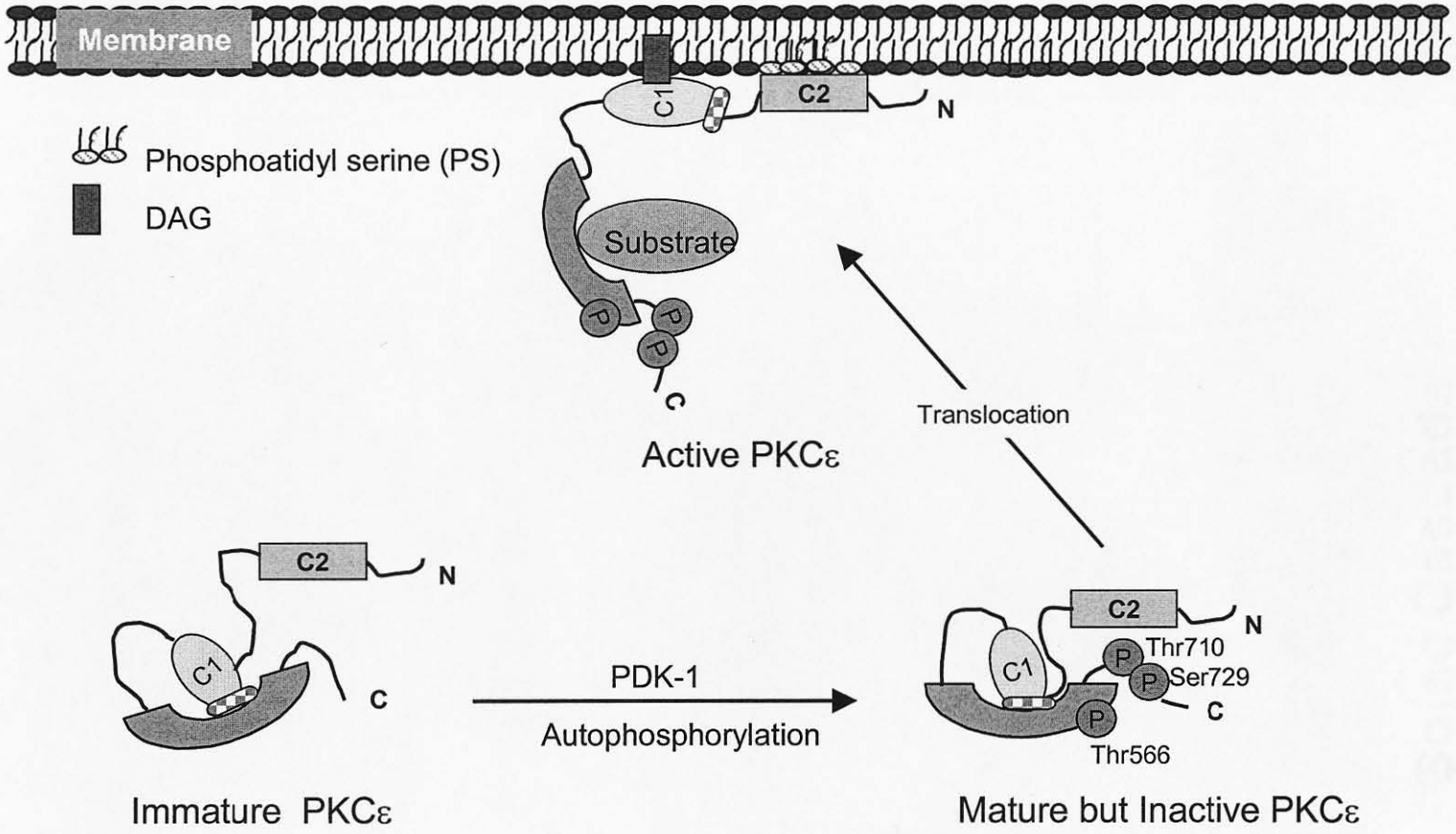
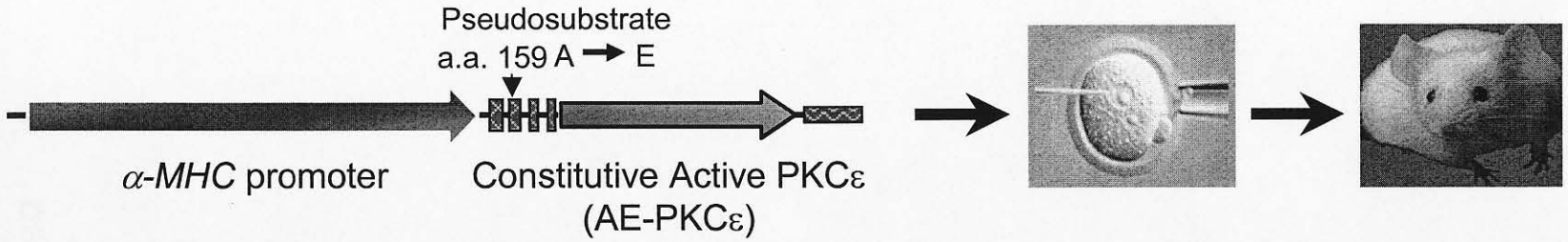

Membrane
Phosphoatidyl serine (PS)
DAG
C1
C2
N
Substrate
P
P
P
C
Active PKCε
Translocation
C2
N
C1
C
PDK-1
Autophosphorylation
C2
N
C1
P
Thr710
P
Ser729
C
P
Thr566
Immature PKCε
Mature but Inactive PKCε

Figure 4. Activation of PKCε reduces myocardial infarction sizes. PKCε transgenic mice was generated using α-MHC cardiac-specific promoter. The DNA constructs used in the generation of constitutively active PKCε mutant transgenic mice has a point mutation at the pseudosubstrate region (A to E at aa159). This mutation would release psudosubstrate domain from catalytic site, thus placing PKCε in its open, and therefore active confirmation When these mice were subjected to myocardial ischemia, the PKCε transgenic mice show significantly decreased myocardial infarction size when compared with non-transgenic mice, indicating that active PKCε protect the heart.


Pseudosubstrate
a.a. 159 A → E
α-MHC promoter
Constitutive Active PKCε
(AE-PKCε)

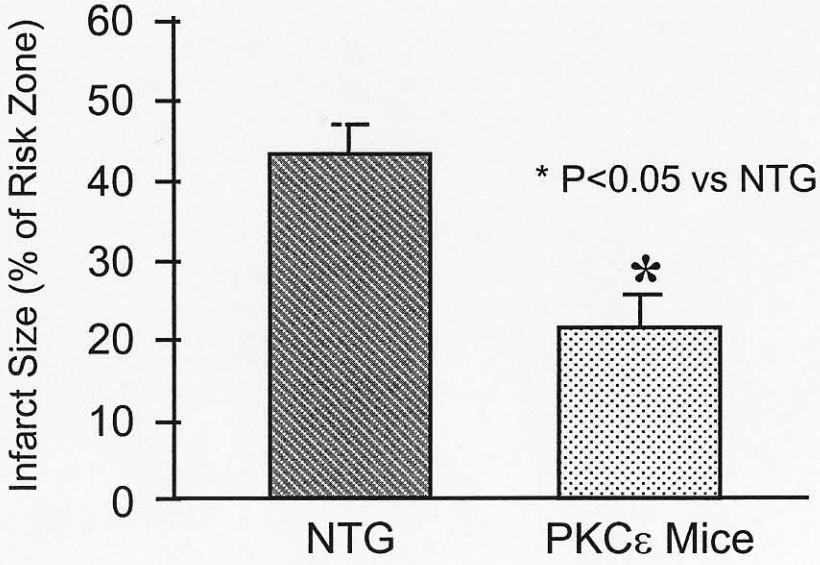
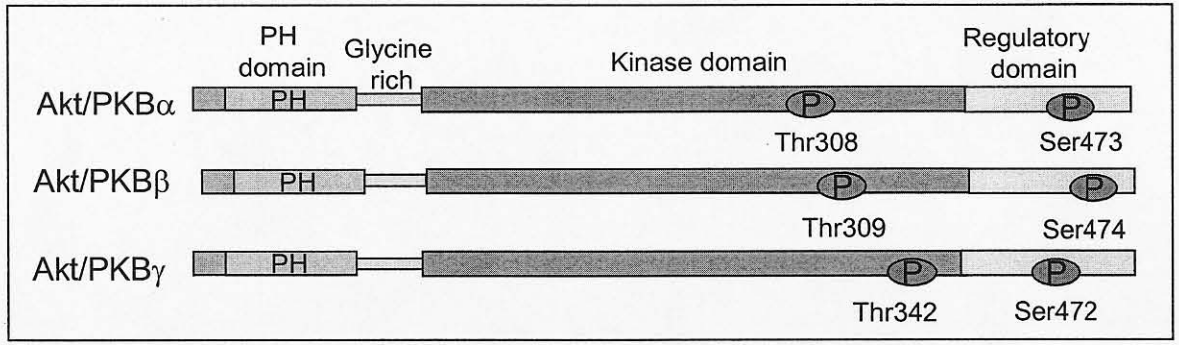

Infarct Size (% of Risk Zone)
60
50
40
30
20
10
0
* P<0.05 vs NTG
*
NTG
PKCε Mice

Figure 5. Molecular structures and activation model of Akt/PKB isoforms. Upper panel, Akt is also a serine/threonine kinases with three major isoforms. Each isoform consists of an N-terminal PH domain, a kinase domain and a C-terminal regulatory domain. N-terminal PH domain can mediate lipid-protein or protein-protein interactions. Central kinase domain has a conserved phosphorylation site Thr308 which is necessary for Akt activation. The hydrophobic C-terminal regulatory domain containing a second regulatory phosphorylation site Ser473, which is required for maximal Akt activation. Lower panel, inactive Akt mainly resides in the cytosol, and the PH domain is serving as an autoinhibitory motif to block kinase domain. (like PS for PKCε). The activation of Akt requires the binding of PIP_3 to PH domain to unmask the kinase domain, leading to the translocation to membrane fraction; Akt activation also requires the phosphorylation modifications of Thr308 and Ser473, which is mediated by kinase called PDK1 and unknown kinases called PDK-2.


PH domain
Glycine rich
Kinase domain
Regulatory domain
Akt/PKBα
PH
P
Thr308
Ser473
Akt/PKBβ
Thr309
Ser474
Akt/PKBγ
Thr342
Ser472

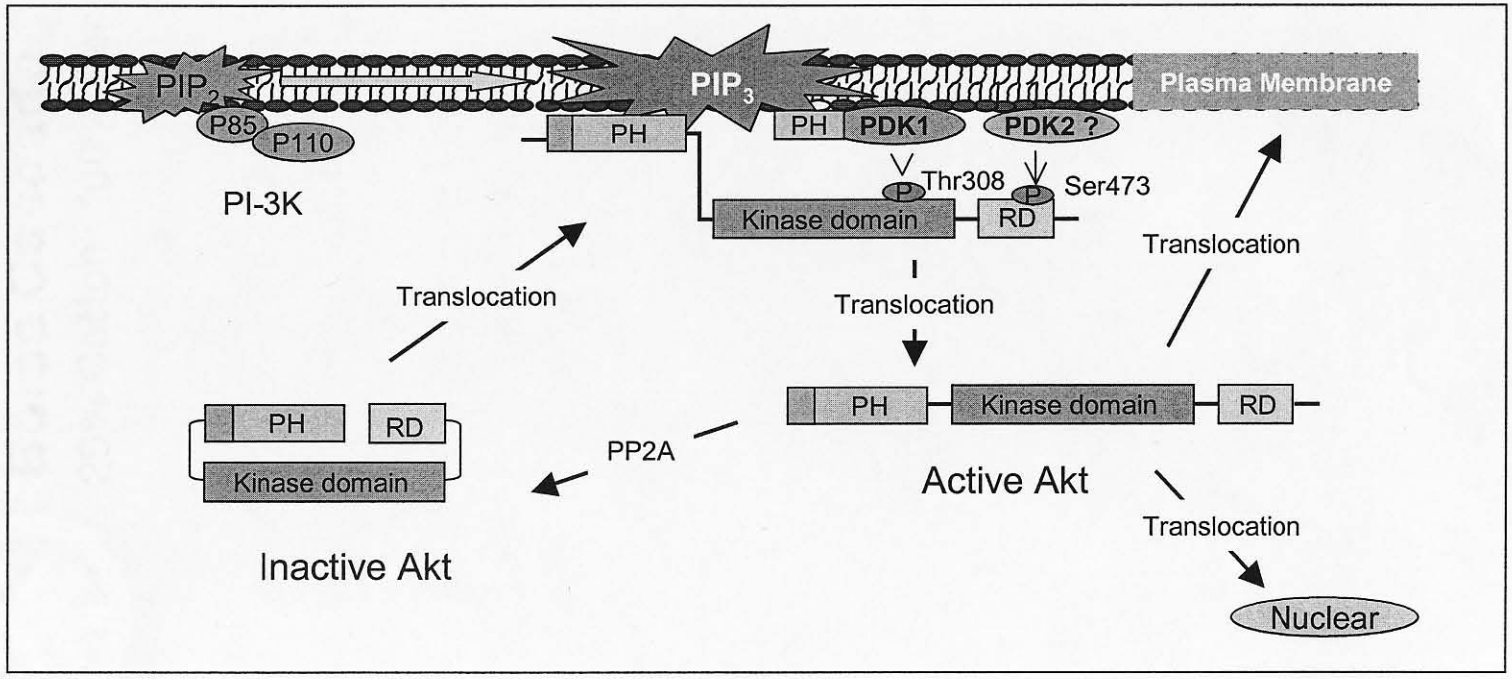
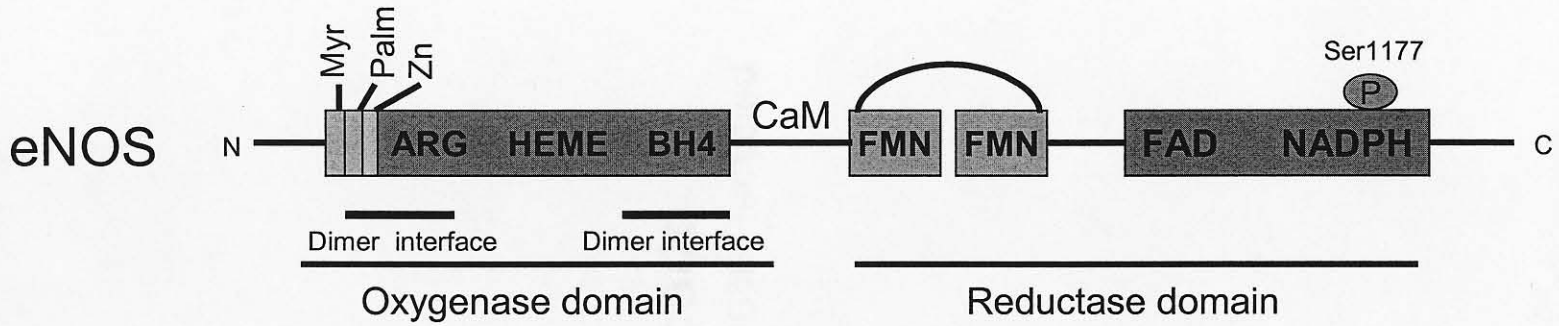
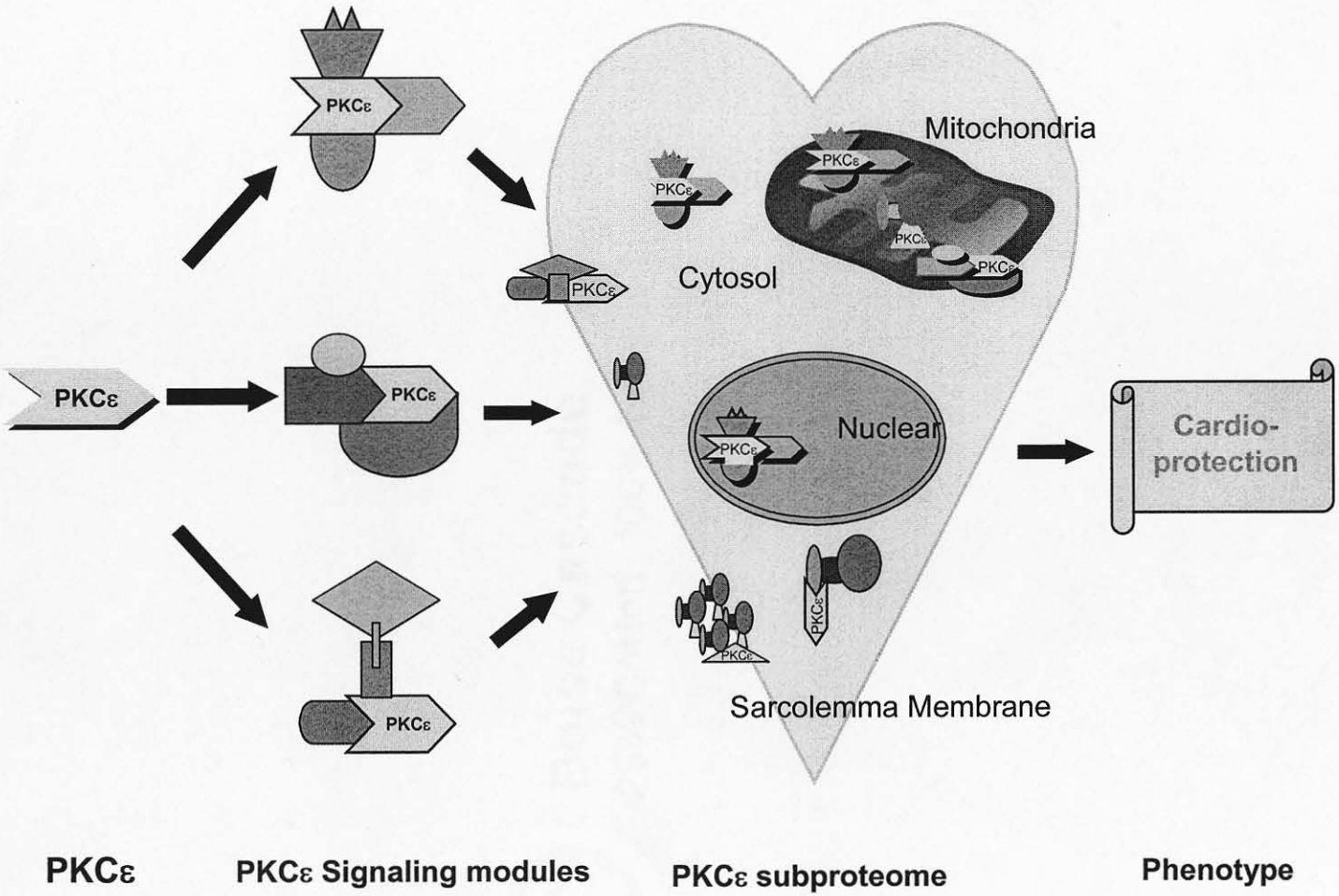

PIP2
PIP3
Plasma Membrane
P85
P110
PI-3K
PH
PDK1
PDK2 ?
Thr308
Ser473
Kinase domain
RD
Translocation
Translocation
Translocation
Translocation
PH
RD
Kinase domain
PP2A
Active Akt
Inactive Akt
Nuclear

Figure 6. Molecular structure of endothelial nitric oxide synthase (eNOS). eNOS is Ca^{2+}-CaM-dependent nitric oxide syntheses, and has two functional domains. C-termianl reductase domain and N-terminal oxygenase domain. The myrostoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown (important features for eNOS membrane localization), and the autoinhibitory loop within the FMN regions of eNOS is also shown. eNOS can catalyze arginine to produce nitric oxide. And its activity is highly regulated by phosphorylation of Ser1177, resulting in an increased activity and increased NO production.



L-Arginine + O_2 ⟹ Citrulline + NO or NO^-

Figure 7 Architecture of the cardioprotective PKCε signaling system in cardiac myocytes. The hypothetical structure of the PKCε signaling subproteome involves various signaling modules located at multiple subcellular compartments, and their integrity carries out signal transduction tasks required for cardioprotection.


PKCε
PKCε
PKCε
PKCε
PKCε
Mitochondria
PKCε
PKCε
PKCε
PKCε
Cytosol
Nuclear
PKCε
Cardio-
protection
PKCε
PKCε
PKCε
Sarcolemma Membrane
PKCε
PKCε Signaling modules
PKCε subproteome
Phenotype

CHAPTER II

METHODS AND MATERIALS

All procedures were performed in accordance with the Animal Research Committee guidelines at UCLA, which conform with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

Antibodies and Chemicals

Anti- β'-adaptin, BMX, caveolin-3, connexin-43, COX-2, desmin, ERK1, HIF-1α, GM130, Lap2, MEK1, eNOS, HSP60, iNOS, NuMA, p38 MAPK, p85 PI-3K, p170 PI-3K, PKCε, Pyk2, Ras, RACK1, and villin antibodies were purchased from Pharmingen (San Diego, CA). Anti-AKAP79, Akt, ANT1, c-Fos, c-Jun, c-Myc, CREB, Elk, HO-1 HSP27, HSP70, GRP75, hnRNPa2/b1, JNK2, lamin-C, Lck, NF-κB (p65), PDK1, Src, STAT1 and 3, Tropinin I and T, vimentin, vinculin, Akt1/2, Akt1, Akt2 and Akt3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-αB-crystallin and TCP-α (RACK2) antibodies were purchased from Stressgen (Vancouver, BC). Anti-phospho-Akt of Ser308 and Ser 473, anti-phospho-eNOS (Ser1177) antibodies and Akt kinase assay kit were from Cell Signaling Technology Inc. (Beverly, MA). Anti-α-actin and α-actinin antibodies from Sigma-Aldrich (St. Louis, MO). Anti-Na^+/K^+ ATPase, histone H4, PP2A, PP1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), anti-prohibitin-1 antibody from Research Diagnostics (Flanders, NJ), and anti-VDAC1 from calbiochem (San Diego, CA). Anti-ribonucleoprotein (RNP) antibody was from NeoMarkers (Fremont, CA), anti-glyceraldehydes-3-PDH (GAPDH) antibody was from Biodesign (Saco, Maine), and anti-creatine kinase was from abcam (Cambridge, UK). The aldose reductase antibody was a generous gift from Dr. Aruni Bhatnagar (University of Louisville). The hnRNPH1

antibody and hnRNP K antibody were generous gifts from Dr. Doug Black (Howard Hughes Medical Institutes, CA), and Dr. Pradip Raychaudhuri (University of Illinois at Chicago), respectively. The Oracle antibody was custom made by BioSource (Camarillo, CA). Cross-linker EZ-Link Sulfo-NHS-LC-Biotin and streptavidin:HRP were obtained from Pierce (Rockford, IL). L-arginine, Bistris, tricine, sucrose, 6-aminocaprioc acid, and other chemicals were purchased from Sigma-Aldrich. Recombinant active Akt, unactive Akt, PH domain of Akt, PH domain deleted Akt were purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant PKCε and eNOS were bought from Biomol (Plymouth meeting, PA) and Calbiochem (La Jolla, CA), respectively.

PKCε Transgenic Mice

The cardiac-targeted PKCε transgenic mice used in these studies have been previously described (142, 145). This transgenic line exhibits moderate increases in PKCε activity, is inherently cardiac protected, and is free from hypertrophy. Transgenic mice and their non-transgenic littermates were used at 9-12 weeks.

Subcellular fractionation

All fractionation procedures were performed at 4°C. Myocardial cytosolic, nuclear, mitochondrial, membrane fractions were obtained by differential centrifugation as previously described (12). Briefly, mouse hearts were homogenized in buffer containing 250m*M* sucrose, 10m*M* Tris-HCl (pH7.4), 1m*M* EDTA, 1m*M* Na_3VO_4, and a cocktail of protease inhibitors (Roche, Indianapolis, IN). The homogenate was then centrifuged at 1000 x g for 10 min to pellet the nuclear fraction. The supernatant was centrifuged at 10,000 x g for 30 min to yield the mitochondrial fraction in the pellet (12). The resultant supernatant was subsequently centrifuged at 100,000 x g for 1 hr to yield the cytosolic fraction (supernatant) and membrane fraction (pellet). The different membrane fractions were then re-suspended in homogenization buffer with 0.5% (v/v) NP-40. Caveolae were isolated by differential density centrifugation, as described by Smart *et al* (159).

Immunoprecipitation

PKCε monoclonal antibodies were covalently cross-linked to protein-A/G beads (Santa Cruz) using a commercially available kit (Pierce). Subcellular lysates (1000μg) were incubated with the immobilized PKCε antibody overnight at 4°C and washed three times with buffer containing 150 m*M* NaCl, 20 m*M* Tris-HCl (pH 7.4), 10 m*M* EDTA, 1 % (v/v) NP-40, 1 m*M* Na_3VO_4, and a protease inhibitor cocktail (Roche). After the final wash, the beads were re-suspended in Laemmli buffer, boiled for 10 min, and then the supernatants were subjected to SDS-PAGE and immunoblotting. If immunoprecipitation was followed by two dimensional (2D) electrophoresic analysis, after the final wash, the beads were washed with 10% sucrose to remove salt, and the protein complexes then dissolved into thiourea buffer (Urea 7*M*, thiourea 2*M*, CHAPS 2% (w/v), DTT 65m*M*, Zwittergent 1% (v/v), Ampholytes (pH3-10) 0.8% (w/v), and bromphenol blue 0.01% (w/v) (Genomic Solutions, Ann Arbor, MI).

Immunoblotting

Samples were denatured in Laemmli buffer, resolved by one-dimensional SDS-PAGE, and transferred onto nitrocellulose membranes. After blocking with 5% (w/v) nonfat milk powder, the membranes were immunoblotted using the ECL detection system (Amersham, Piscataway, NJ).

Two-Dimensional Electrophoresis

Subcellular extracts were loaded onto immobilized pH gradient (IPG) strips (Amersham) and subjected to isoelectric focusing in the first dimension as described (142). The IPG strips were re-equilibrated and subjected to SDS-PAGE (Genomic Solutions) for the second dimension. Proteins were then visualized by silver staining.

Protein Biotinylation

Following PKCε immunoprecipitation, protein complexes were eluted with low pH elution buffer (Pierce) and then dialyzed against PBS overnight at 4°C. To biotinylate the proteins, the dialyzed sample was incubated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) at 4°C for 4 hours. The

samples were then subjected to SDS-PAGE, transferred to nitrocellulose membranes, and visualized using streptavidin-HRP (Pierce) and the ECL system (Amersham).

Gel Filtration Chromatography

Gel filtration chromatography was carried out as described (36) with a few modifications Subcellular fractionations which were prepared from 15 hearts in Buffer A containing: 20m*M* HEPES, pH 7.9, 1.5m*M* $MgCl_2$, 150m*M* NaCl, 0.2m*M* EDTA, 0.5% (v/v) NP-40, and a cocktail of protease inhibitors, or total homogenates from two mouse hearts were dialyzed overnight at 4°C in Buffer A without NP-40, and clarified prior to chromatography. These samples were then loaded onto a pre-calibrated Sephacryl S-400 column (XK 26/70, 26 mm diameter, 70 cm length, Amersham) using Buffer A without NP-40 as running buffer. Each 1ml fraction was collected. Proteins were then precipitated with cold acetone, and analyzed by either SDS-PAGE or native gel electrophoresis. Thyroglobulin (669kDa), Ferritin (440kDa), catalase (232kDa) and aldolase (158kDa) (Pharmacia) were used as molecular standards.

Native Gel Electrophoresis

Native gel electrophoresis was carried out as described (153, 154) with minor modifications. In particular, deoxycholic acid was used in place of Coomassie blue. Gel filtration fractions were precipitated with cold acetone and then solubilized in buffer containing 50m*M* 6-aminocaproic acid, 200m*M* Bis-Tris, pH 7.0, 20% (v/v) glycerol, and 0.5% (v/v) NP-40 for 1 hour on ice. Then samples were mixed with 10x native sample buffer containing 100m*M* Bis-Tris, pH 7.0, 1*M* 6-aminocaproic acid, 35% (v/v) glycerol, and 1.0% (v/v) deoxycholic acid. After clarification, the samples were loaded onto 5-13% gradient native gels. Electrophoresis was performed at 100 Volts with 15mA per gel overnight at 4°C. Thyroglobulin (669kDa), Ferritin (440kDa), and catalase (232kDa) (Pharmacia) were used as molecular standards.

GST-PKCε Pull-Down

GST-PKCε affinity pull-down assays were performed as previously reported (142). Briefly, GST-PKCε recombinant protein was generated using the baculovirus system (Pharmingen). Subcellular fractions were incubated with GST-PKCε in binding buffer containing 0.5% (v/v) Triton X-100, 20m*M* Tris-HCl, pH7.4, 150m*M* NaCl, 1m*M* EDTA, 1m*M* EGTA, and a cocktail of protease inhibitors (Roche) overnight at 4°C. The beads were washed three times and the proteins eluted with glutathione (GSH) elution buffer (Pharmingen).

Sucrose Gradient Purification

Proteins eluted from the GST-PKCε pull-down were overlayed on a 10-30% sucrose gradient (132). Eighteen consecutive fractions, beginning from the top, were collected. Thyroglobulin (669kDa) and catalase (232kDa) were used as molecular standards. Protein fractions were precipitated with cold 10% (w/v) TCA and 90% (v/v) acetone, resolved by SDS-PAGE, and visualized by silver staining. To identify PKCε signaling complexes, the SDS-PAGE was blotted by PKCε antibodies.

Phosphorylation of Akt or eNOS by PKCε

Recombinant Akt or eNOS was incubated with PKCε in PKC reaction buffer containing 0.03mg/ml Lα-phosphatidyl-L-serine, 2.5μg/ml phorbol 12-myristate 13-acetate, 3.5mM dithiothreitol (DTT), 100μM ATP, 6.5mM magnesium chloride, 50mM Tris-HCl, pH7.5 at 30°C for 30min. The reaction was terminated by addition of 5×Laemmli sample buffer and boiled 5 min at 90°C. The proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE), dried and exposed to X-ray films.

Akt Activity Assay

Akt kinase activity assay was measured using a commercial available kit from Cell Signaling. Briefly, Akt immunoprecipitant were incubated with 1μg GSK-3 fusion protein in the presence of

ATP and kinase assay buffer containing 25mM Tris·HCl (pH 7.5), 5mM β-glycerolphosphate, 2mM dithiothreitol, 0.1mM Na_3VO_4, 10mM $MgCl_2$ at 30°C for 30 min. Phosphorylation of GSK-3 is measured by Western blotting using a phosphor-GSK-3 (Ser21/9) antibody.

eNOS Activity Assay

eNOS activity was assayed via measuring NO_2/NO_3 production using colorimetric assay kit from Calbiochem. Briefly, incubate recombinant eNOS with/out pretreatment or eNOS immunoprecipitant from mouse heart with reaction buffer containing 1.25mM NADPH, 1.25mM arginine, 0.5mM $CaCl_2$, 3μM tetrahydrobiopterin (BH4), 1μM flavin adenine dinucleotide, 1μM flavin adenine mononucleotide and 25mM Tris-HCl, pH7.4 for 30 min at 30°C. Then nitrate was conversed to nitrite with nitrate reductase treatment. Total nitrite was measured by using the Griess reaction as modified by Gilliam et al (68).

Statistical Analysis

Data are reported as mean±SEM. Differences among the 4 experimental groups were analyzed using one-way ANOVA. If the ANOVA showed an overall significance, post hoc contrasts were performed with Student t tests for unpaired data using the Bonferroni correction (174).

CHAPTER III

Functional proteomic mapping of the myocardial PKCε signaling subproteome in the mouse heart: a subcellular compartment study

Abstract

Functional proteomic analyses have revealed that protein kinase Cε (PKCε), a critical mediator of cardiac protection, forms multiprotein signaling complexes in the myocardium. We previously characterized at least 93 PKCε-associated proteins in the PKCε signaling subproteome including structural proteins, signaling molecules, stress-activated proteins, transcriptional/translational factors, metabolism-related proteins, and PKC-binding domain containing proteins. However, these studies were performed in the context of the whole myocyte, and information regarding the subcellular distribution of these proteins, which is essential for the construction of a cellular map of this subproteome, was lacking. The present studies were designed to investigate the localization and composition of PKCε complexes in each subcellular compartment and the putative mechanisms that govern their assembly. Accordingly, we examined three critical aspects of multiprotein complexes in the myocardium: the molecular size of native protein complexes; the molecular composition of individual protein complexes, and the assembly of each complex in various cellular compartments. Furthermore, the functional significance of these complexes was assessed by delineating their formation in the genesis of a cardioprotective phenotype. Using a comprehensive functional proteomic strategy that combines various protein isolation, separation, and identification techniques, we found that: 1) the cardiac PKCε signaling subproteome is comprised of various multiprotein PKCε complexes; 2) the native size of each PKCε complex differs over a wide range of molecular weight among cytosolic, membrane, nuclear,

mitochondrial, and caveolar compartments; 3) different PKCϵ complexes appear to have distinct molecular compositions, which vary with subcellular location, suggesting that subcellular location contributes to the identity of PKCϵ-associating proteins; and 4) the assembly of these complexes is dynamically modulated in the cardioprotected when compared with the normal heart. Taken together, our data represent the first comprehensive analysis of subcellular PKCε protein complexes and demonstrate that formation of these signaling complexes is subcellular compartment-specific.

Introduction

Molecular interactions among proteins serve as essential mechanisms to facilitate the transmission of messages within the cell. Functional proteomic analyses of several signal transduction systems in various cell types have advanced our understanding of how such messages are relayed (91, 97-99, 113, 142, 168, 173). Whereas the majority of previous studies have examined the functional interaction of only limited molecules, proteomic analysis of an entire subproteome enables delineation of complex molecular interactions in both normal and diseased states, providing a more comprehensive picture of how a given signaling network operates. For instance, an investigation by Husi *et al.* identified 77 proteins within the brain NMDA receptor complex, which include cytoskeletal, adaptor, and signaling molecules (98, 99). In a different study, several proteins, ranging from DNA repair to cytoskeletal regulation, were found to undergo expression and/or post-translational alterations following either activation or inhibition of the serine/threonine protein kinase MEK, a key component of the ERK pathway (113).

Recently, our laboratory has used functional proteomics to study myocardial signaling (52, 142). These analyses of the cardiac PKCε signaling subproteome revealed that PKCε forms multimeric signaling complexes with at least 93 proteins, which can be categorized as structural proteins, signaling molecules, stress-responsive proteins, metabolism-related proteins, transcription- and translation-related proteins, and PKCε binding domain-containing proteins (52, 142). Moreover, cardiac protection facilitated by transgenic activation of PKCε was concomitant with altered recruitment and post-translational modification of many protein residents within the PKCε complexes. Together, these studies indicate that a given signaling element, such as PKCε, can interact with and modulate a wide range of proteins, diverse in both structure and biological function.

Recent studies have demonstrated that subcellular compartmentalization is a critical

factor in defining signaling specificity (97, 173). Therefore, the delineation of a detailed cellular map of a signaling system would be of essential importance to our understanding on how this system supports a biological function. Using whole cardiac cell lysates (52, 142), our previous studies identified many molecular participants that comprise the cardiac PKCε signaling subproteome, however, information regarding the subcellular localization of PKCε associated proteins, which is essential to the mapping of this subproteome, is unknown. The formation and activation of specific PKCε signaling complexes at different subcellular compartments has been implicated to have significant functional consequences (12, 172, 173). Characterization of the subcellular distribution of PKCε complexes should provide key insights into the signaling mechanisms underlying the cardiac phenotypes observed in response to PKCε activation.

Accordingly, in the present study, a systematic examination of PKCε complexes was conducted in the cytosolic, nuclear, mitochondrial, membrane, and caveolar subcellular fractions of mouse hearts, with regard to both their native sizes and their protein compositions. Moreover, the dynamic modulation of each subcellular PKCε signaling complex in response to a phenotypic change was also assessed using cardiac protected PKCε transgenic mice that have been previously established in our laboratory (Figure 8). Our data provides critical information to the mapping of the cardioprotective PKCε signaling subproteome in the murine myocardium.

Results

Subcellular fractionation of mouse hearts and isolation of multiprotein PKCε complexes. Mouse hearts from transgenic PKCε (TG) and nontransgenic (NTG) littermates were homogenized and the lysates were fractionated by differential centrifugation methods into cytosolic, nuclear, mitochondrial, membrane and caveolar fractions. 2D electrophoresis followed by silver staining revealed largely disparate patterns of proteins among various subcellular fractions (Figure 9). To verify the subcellular fractionation processes, immunoblotting for subcellular compartment-specific proteins was carried out for each fraction. The nuclear-specific protein NuMA was only found in the nuclear fraction, whereas the sarcolemmal Na^+/K^+ ATPase was primarily localized in the membrane fraction, and the mitochondrial protein prohibitin-1 was mainly concentrated in the mitochondria (Figure 10). Finally, cytosolic separation was assessed using lactate dehydrogenase (LDH) activity, with >98% of the total LDH activity residing in the cytosolic fraction, and only ≤0.85% activity present in any of the other cellular fractions (Figure 10). Therefore, our subcellular fractionation procedure was largely efficient with minimum cross-contamination.

We next examined PKCε expression in each subcellular fraction from both NTG and TG hearts by immunoprecipitating PKCε and then immunoblotting for PKCε. Figure 11 shows that PKCε could be efficiently immunoprecipitated from each fraction, which enabled isolation of multiprotein PKCε complexes for further studies in a subcellular-specific manner. As expected the levels of PKCε in every compartment were considerably higher in the TG mouse hearts when compared with the NTG mouse hearts.

Following immunoprecipitation, multiprotein PKCε complexes from different subcellular fractions were eluted and visualized by either 1D SDS-PAGE (Figure 12) or 2D PAGE (Figure 13) followed by silver-staining. Figure 12 illustrates significant differences in PKCε-associated

proteins in the cytosolic, nuclear, mitochondrial, and membrane subcellular compartments. These differences include the number of proteins present within each subcellular PKCε complex as well as the molecular weights of proteins within each subcellular PKCε complex. A similar trend was found following 2D separation, examples of which are shown in Figure 13. Multiprotein PKCε complexes from cytosolic and nuclear fractions displayed distinct 2D electrophoretic patterns of protein spots. Taken together, our results show that each subcellular fraction contains PKCε signaling complexes. Moreover, our data show that the number and size of the proteins within the PKCε complexes differs in each subcellular compartment. The nature of such differences highlights potential subcellular-specific differences in PKCε dependent signal transduction and cellular function.

Delineation of the physical sizes of native multiprotein PKCε complexes.

Having validated the subcellular fractionation and complex isolation procedures, large-scale homogenization and fractionation of NTG mouse hearts were performed. Subcellular protein mixtures from 15 hearts were analyzed by gel filtration chromatography using a Sephacryl S400 column to separate intact protein complexes on the basis of their physical size. The presence of multiprotein PKCε complexes in the different subcellular fractions was then determined by PKCε immunoblotting. Figure 14 shows the chromatographic profiles of PKCε protein complexes in cytosolic, membrane, mitochondrial, and nuclear fractions, respectively. Although a significant amount of PKCε migrated in the form of either a monomer or a dimer (elution volumes 250-280ml), especially in the cytosolic and nuclear fractions, the migration pattern of PKCε at lower elution volumes demonstrated that PKCε associates with many other proteins in different sized complexes. The most striking finding was that the elution profiles of PKCε protein complexes were very different in each subcellular fraction, again emphasizing the heterogeneity of multiprotein PKCε complexes, both in size and composition, among compartments.

Individual fractions separated by gel filtration were further analyzed for PKCε complex formation. After immunoprecipitation with PKCε antibodies, PKCε complex proteins were eluted

and cross-linked with a Sulfo-NHS-LC-Biotin conjugating agent. The biotinylated proteins were then separated by 1D SDS-PAGE and visualized by immunoblotting using a streptavidin-HRP conjugate (Figure 15a). The results confirmed that PKCε associates with a battery of proteins in the mouse heart. Again, it was evident that the collection of proteins within the PKCε complexes differed across individual subcellular compartments. As expected, the larger complexes were found in the lower elution volumes and contained more components in general; whereas the smaller complexes were found in the higher elution volumes and hosted fewer proteins.

To insure that our findings regarding these complexes were not due to protein aggregation artifacts, we performed additional experiments to study these multiprotein complexes using recombinant GST-PKCε as bait proteins. Cardiac subcellular fractions were incubated with GST-PKCε fusion proteins and allowed to form complexes. These complexes were then isolated from the cell lysate by GST pull-down and further fractionated by sucrose gradient centrifugation. Gel analysis and immunoblotting of sucrose gradient fractions showed that in all subcellular compartments (Figure 15b shows cytosolic as an example), GST-PKCε forms specific multiprotein complexes of varying sizes. The peak sedimentation fraction for PKCε appeared to migrate at ~200kDa; however, PKCε containing fractions were also traced at higher weights (>669kDa), showing that large complexes were also formed (Figure 15b). In further complex formation tests, analysis of two selective fractions from gel filtration by deoxycholate-buffered native gel separation definitively show that PKCε is present in several high molecular weight complexes (>880kDa). Combined with the expression profile of each subcellular fractionate generated by gel filtration, these data show that PKCε forms numerous complexes in each compartment that are largely heterogeneous in size and molecular weight (Figure 15c).

Characterization of protein compositions of subcellular PKCε complexes in the mouse heart

In order to assess the identity of the proteins within the subcellular PKCε complexes, PKCε was immunoprecipitated from each fraction and the complexes analyzed by immunoblotting for proteins previously demonstrated to be components of the total cardiac PKCε complex (52, 142).

Protein expression profiles of the PKCε signaling complexes in cytosol

The number of proteins constituting the cytosolic PKCε signaling complex was extensive (Table 1). Many contractile proteins found in the soluble fraction, such as troponin I and actin, resided in the PKCε complex. Interestingly, RACK2 and AKAP79, which are anchoring proteins for several kinases, including PKC, were also associated with cytosolic PKCε complexes. Moreover, many signaling proteins interacted with cytosolic PKCε including lipid kinases (e.g. PI 3-kinase) (Figure 16), tyrosine kinases (e.g. Lck, Pyk2), serine/threonine kinases (e.g. Akt, MAPK), and protein phosphatases (e.g. PP1, PP2A). Stress-activated proteins involved in the synthesis of low molecular weight mediators such as nitric oxide (eNOS) (Figure 16), prostaglandins (COX2), and carbon monoxide (HO-1) were also key components, alongside several members of the heat shock protein family (HSP70, HSP27). The hypoxia sensitive transcription factor Hif-1α was also found in the cytosolic complex, consistent with the cytoplasmic localization of Hif-1α in quiescent cells (37), as were multiple metabolic proteins, such as creatine kinase.

Protein expression profiles of the PKCε signaling complexes in nucleus

The nuclear PKCε complex was as extensive in composition as the cytosolic fraction (Table 1). Not surprisingly, the nuclear proteins LAP2, NuMA, and lamin C bound to PKCε in this fraction. A similar profile of signaling kinases and stress-activated proteins that was seen in the cytosol was also observed in the nuclear signaling complex. However, unlike the soluble fraction, we also found the tyrosine kinases Src and BMX in the nuclear PKCε complex. In addition, multiple transcriptional proteins resided in the nuclear PKCε complex, including c-Fos, c-Jun, NF-κB, Elk-1, CREB, and STATs.

Protein expression profiles of the PKCε signaling complexes in membrane

Consistent with the hypothesis that RACK proteins mediate PKCε translocation to membranous structures, we found both RACK1 and RACK2 in the membrane PKCε complex (Table 1).

AKAP79 also co-localized with PKCε complex in this compartment. Other structural proteins in the membrane complexes included the intermediate filament protein desmin, the tight junction protein villin, the Golgi matrix protein GM130, and β'-adaptin, a constituent of clathrin-coated pits. Along with the many kinases and stress-activated proteins already observed in the cytosolic and nuclear complexes, the cardiac-specific gap junction protein connexin-43 also interacted with PKCε in the membrane.

Protein expression profiles of the PKCε signaling complexes in caveolae

Although the cytosolic, nuclear, and membranous PKCε signaling complexes contained a large number of molecules, we found that the expression profile of proteins in the caveolar PKCε complex was by comparison limited (Table 1). The structural protein caveolin-3 was a major component of the PKCε complex in this specialized micro-domain. RACK2 and β'-adaptin were also found. Again, several kinases were associated with PKCε in caveolae including Ras, and Akt, the tyrosine kinases Src and Lck, and all three MAPK families. In agreement with previous investigations demonstrating strong caveolar localization of NOS (80, 151), both eNOS and iNOS interacted with PKCε in mouse cardiac caveolae (Figure 7).

Protein expression profiles of the PKCε signaling complexes in mitochondria

Our initial analysis revealed number of PKCε associating proteins in the mitochondrial fraction (Table 1). Notably that these proteins include the mitochondrial import protein, prohibitin-1, and the mitochondrial metabolic proteins ANT, VDAC, and isocitrate dehydrogenase. In addition, the signaling kinase profile of the mitochondrial PKCε complex contained multiple tyrosine kinases, as well as members of the MAPK and Akt signaling pathways. There were also a number of stress proteins associating with PKCε in the mitochondria, all pertaining to mediator synthesis and metabolism, such eNOS (Figure 7) and COX2.

Modulation of subcellular PKCε complexes size in protected myocardium.

It has been previously demonstrated that cardiac-specific transgenic activation of PKCε renders the mouse heart resistant to ischemia/reperfusion injury (136, 142, 145). In addition, analysis of the PKCε complexes from the total cell lysates of protected hearts and wild type mouse hearts showed alterations in the interaction of many proteins with these complexes (52, 142). However, it remained unknown what mechanisms regulated the association of given proteins with PKCε. Thus, the present study investigated the role of subcellular location as a regulatory determinant of the formation of these complexes during phenotypic changes. Gel filtration experiments were performed using PKCε TG mice hearts and then compared to those conducted with NTG mouse hearts to determine the alterations of native subcellular PKCε complexes during cardiac protection. As seen in Figure 6, the native protein profiles of PKCε complexes in TG hearts were also subcellular compartment-specific. In general, the size of native PKCε complexes were increased in the PKCε cardioprotected hearts, with a concomitant decrease in the free forms, that is, the monomeric and dimeric forms, of PKCε, further supporting the concept that protective PKCε signaling involves the recruitment of proteins into these complexes. In particular, in the cytosolic fraction, PKCε formed putatively several very high molecular weight (>MDa) complexes in the TG heart that were not present in the NTG heart. The mitochondrial, membrane, and nuclear PKCε profiles also exhibited dramatic changes in the TG mouse hearts compared with those in NTG hearts. Taken together, these data indicate that PKCε recruits different molecules into the complexes depending on the subcellular location and that the subcellular complexes are individually modified in the protected versus the naïve heart. This finding suggests that this subcellular organization of multiprotein signaling complexes contributes to the simultaneous modulation of multiple functions that lead to a given phenotype, in this case cardiac protection.

In addition to modulation of native PKCε complex size, the composition of each PKCε complex was altered in a subcellular-specific manner in the TG mouse hearts. Indeed, striking variation in the expression profiles of PKCε complexes was observed across different cardiac subcellular locations when normal hearts were compared to those protected against ischemic

injury (Table 2). Enhanced binding of a variety of signaling and stress-activated molecules to PKCε was for the most part subcellular compartment-specific. For example, eNOS was upregulated in all the subcellular PKCε complexes whereas the 170kDa isoform of PI 3-kinase was only increased in the cytosolic and membrane fractions (Figure 16). Regarding structural proteins, the PKCε-selective receptor protein, RACK2 exhibited greater interaction with PKCε in both the cytosol and the membrane fraction of the TG mouse hearts, whereas interaction of PKCε with the LIM/PDZ-containing protein Oracle, a protein that has been characterized in cardiac development (93, 137, 189), was only elevated in the nuclear fraction. The upregulation of these specific proteins in specific subcellular complexes points to the ability of PKCε to modulate multiple, discrete cellular functions.

Discussion

Although several efforts in whole organelle proteomic analyses of mitochondria (12, 77, 118), Golgi (17, 178) and other subcellular fractions (36, 90, 109, 126, 131, 139) have achieved fruitful results, comprehensive characterization of the entire proteome of a particular organelle remains a Herculean task. Major challenges include the dynamic nature of these organelle proteomes with respect to the changes of time and cellular functions. In contrast, studies focusing on the examination of specific subproteomes, such as the PKCε signaling system, will target a sub-group of proteins on the basis of the specific biological functions they perform and will define temporal alterations of these proteins (during the time course of a phenotype change). Consequently, this approach is more focused and will enable us to circumvent the aforementioned challenges. In accord with this concept, simultaneous proteomic analysis of a particular subproteome in all subcellular compartments will illustrate a cellular map of this subproteome, and would offer important novel insights.

Consequently, the primary objectives of the present investigation were to delineate the subcellular distribution of the PKCε signaling subproteome and to understand how assembly of this subproteome is modulated during cardioprotection. We hereby report that the PKCε signaling subproteome is comprised of various multiprotein PKCε signaling complexes; these complexes differ in their native molecular size, and the formation of these complexes is modulated on a subcellular basis within cardiac cells. To our knowledge, this is the first study to demonstrate the formation of native, signaling kinase (PKCε)-based, multiprotein complexes, and to define how modulation of these complexes directly correlates with a specific phenotype (cardioprotection). This study provides three lines of novel information: 1) these data show differential regulation of multiprotein complex assembly across subcellular locations, suggesting that this subcellular organization of distinct multiprotein complexes may serve as a mechanism for signal transduction; 2) these data demonstrate that a signaling kinase, namely PKCε, can form

protein complexes with numerous other functionally diverse proteins, and that the distinct molecular components of these complexes appear to be modulated in a stimulus and subcellular location-specific manner; and most importantly, 3) these findings suggest that analysis of intact, non-denatured multiprotein complexes is an essential step to further our understanding of the manner in which these complexes transduce signals in normal and diseased organs.

We achieved mapping of the PKCϵ subproteome using two complimentary approaches. The first approach encompasses the biochemical methods of gel filtration, native gels, and 2D electrophoresis, which enabled the determination of the size and composition of the protein complexes. The data presented herein shows clearly that PKCε forms multiprotein complexes that contain many components. Size fractionation of subcellular proteins using gel filtration showed a dynamic size range of PKCε complexes, which differ cross subcellular compartments, and are dynamically modulated in the non-transgenic and cardioprotected PKCε transgenic hearts (Figure 14). Consistent with these findings, GST-PKCε pull-down and sedimentation sucrose gradient analysis illustrate that multiprotein PKCε complexes are heterogeneous and multimeric in nature *in vivo* and *in vitro*. Similarly, the 2D electrophoretic display of PKCε complexes from cytosol and nuclear subcellular fractions yielded different patterns (see Figure 13c) concurrent with the hypothesis that each subcellular fraction contains distinct PKCε complexes.

The second approach taken was the systematic identification of proteins within individual subcellular compartmentalized PKCε complexes, using classical immunoprecipitation and immunoblotting techniques (Table 1 and 2). The majority of previous proteomic studies have relied on mass spectrometry for protein identification. Indeed, using this strategy, our laboratory has previously identified 93 proteins in the PKCε signaling subproteome (52, 142). However, in view of the concerns of the sensitivity of our established mass spectrometry data, we have performed a substantial amount of immunoblotting analyses to verify and substantively confirm these results (52, 142).

The data revealed that the number and nature of specific proteins present within a PKCε complex was dependent upon the subcellular location (see Table 1). For example, the cytosolic

PKCε complexes contained ~50 proteins from all of the functional groups (signaling, structural, stress-activated, transcription, metabolism, and PKC binding domain). In stark contrast, the caveolar PKCε complexes contained only 13 proteins that were primarily signaling and structural molecules; presumably a direct reflection of the specialized nature of this particular subcellular compartment. A somewhat surprising finding was that a large number of PKCε-associated proteins were found outside of their normal subcellular context. The specific PKCε anchor protein RACK2 is thought to localize to cytosolic and membranous compartments such as the sarcolemma and Golgi (41) and indeed co-localized with PKCε in these fractions. However, it was also found to be a key component of the mitochondrial, nuclear, and caveolar complexes. This is an important observation, as it represents a means by which PKCε can localize to these organelles, as has been described during cardiac protection. Similarly, we found that eNOS interacts with PKCε in every compartment (see Figure 16). Many studies have demonstrated the cytosolic and particularly membrane/caveolar localization of eNOS in cardiac cells (24, 180), and thus, the interaction of eNOS with PKCε in these compartments was expected. However, eNOS also co-localized with PKCε in the nuclear and mitochondrial fractions. Nuclear translocation of eNOS in response to VEGF and estradiol (54) has been reported and the formation of nuclear PKCε-eNOS complexes may allow a more direct modulation of transcription by nitric oxide. Nitric oxide is able to permeate the mitochondrial membrane and can influence the function of many components of oxidative phosphorylation (31). Consequently, mitochondrial PKCε-eNOS complexes may enable PKCε to regulate myocardial energy. The functional importance of signaling between PKCε and eNOS has been further elucidated in detail and these findings will be presented in the subsequent Chapter. These examples show that, in addition to further delineating the cardiac PKCε signaling system, the present study provides novel insight into the subcellular expression of a number of molecules and therefore evidence for heretofore unknown functions of PKCε.

The finding that the protein composition of these subcellular PKCϵ complexes was modulated during cardiac protection serves as important evidence for the role of these complexes

to regulate phenotype. As shown in our previous study (142) many of the PKCε associated proteins have been implicated in protection of the heart against ischemic injury cardioprotection. To name a few, Src (146, 160), Lck (144, 145), Akt (39), MAPKs (12, 141), COX2 (78, 158), NOS (78, 181), connexin-43 (47), NF-κB (115, 181), HO-1 (184), and HIF-1 (37) have all been shown to play protective roles in the heart. Interestingly, compartment-specific alterations in the association of these known cardiac protective proteins within PKCε complexes were saliently observed in the present study. For example, recruiting NF-κB to PKCε was only observed in the nuclear fraction whereas increased binding to the caveolin-3 was only found in the caveolar fraction. Again, these data are consistent with the specialized functions of the proteins involved: transcription in the case of NF-κB and the structure/regulation of caveolae in the case of caveolin-3.

In summary, the present study demonstrates that different subcellular locations within the myocardium contain PKCε complexes with various native sizes and distinct molecular components, suggesting that specific cellular tasks at different subcellular locations are facilitated by regulation of these protein complexes. Furthermore, these subcellular complexes are independently modulated in terms of size and composition when the myocardium is protected against ischemic insult. Taken together, these findings support the notion that subcellular-specific organization of multiprotein complexes may serve as a mechanism by which the cell controls its phenotype.

Figure 8. Flow chart for isolation and visualization of PKCε multiprotein signaling complexes – experimental strategy for Chapter III Mouse hearts were homogenized and separated into different subcellular fractions. Then gel filtration chromatography, PKCε immunoprecipitation or GST-PKCε affinity pull down assay were applied to isolate protein complexes. Different methods were subjected to visualize PKCε multiprotein complexes, leading to obtain various data shown in the following figures.

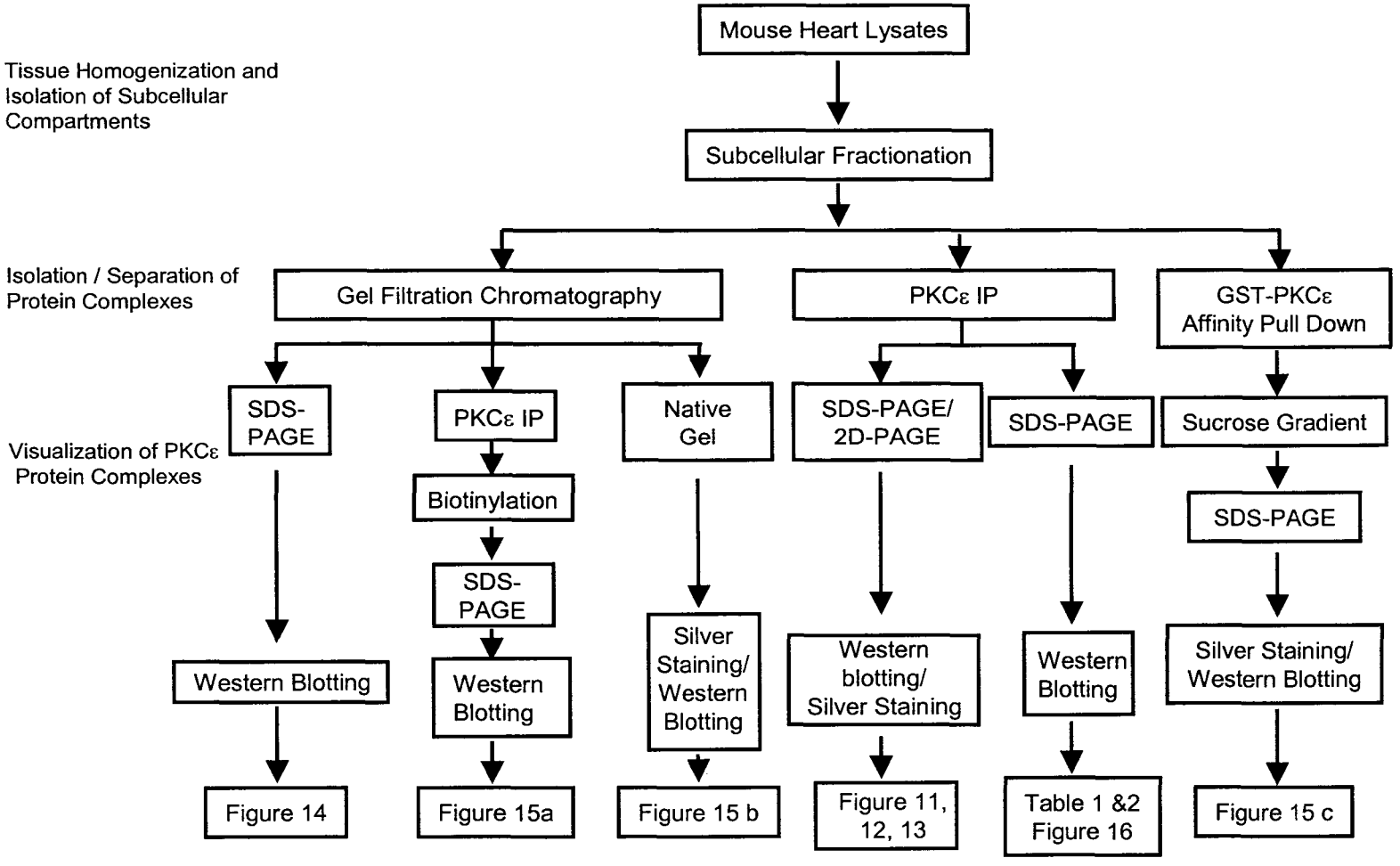

Mouse Heart Lysates
Tissue Homogenization and Isolation of Subcellular Compartments
Subcellular Fractionation
Isolation / Separation of Protein Complexes
Gel Filtration Chromatography
PKCε IP
GST-PKCε Affinity Pull Down
Visualization of PKCε Protein Complexes
SDS-PAGE
PKCε IP
Native Gel
SDS-PAGE/ 2D-PAGE
SDS-PAGE
Sucrose Gradient
Biotinylation
SDS-PAGE
Western Blotting
Western Blotting
Silver Staining/ Western Blotting
Western blotting/ Silver Staining
Western Blotting
Silver Staining/ Western Blotting
Figure 14
Figure 15a
Figure 15 b
Figure 11, 12, 13
Table 1 &2 Figure 16
Figure 15 c

Figure 9. Two dimensional (2D) electrophoresis separating protein mixtures obtained from mouse heart cytosolic (left) and membrane (right) subcellular fractions. The mouse hearts were homogenized and separated into cytosolic, membrane, mitochondrial and nuclear fractions. Subcellular extracts from cytosolic and membrane fractions were loaded onto immobilized pH gradient (IPG) strips and subjected to isoelectric focusing (pI) in the first dimension. The IPG strips were re-equilibrated and subjected to SDS-PAGE for the second dimension by molecular weight (MW). Proteins were then visualized by silver staining

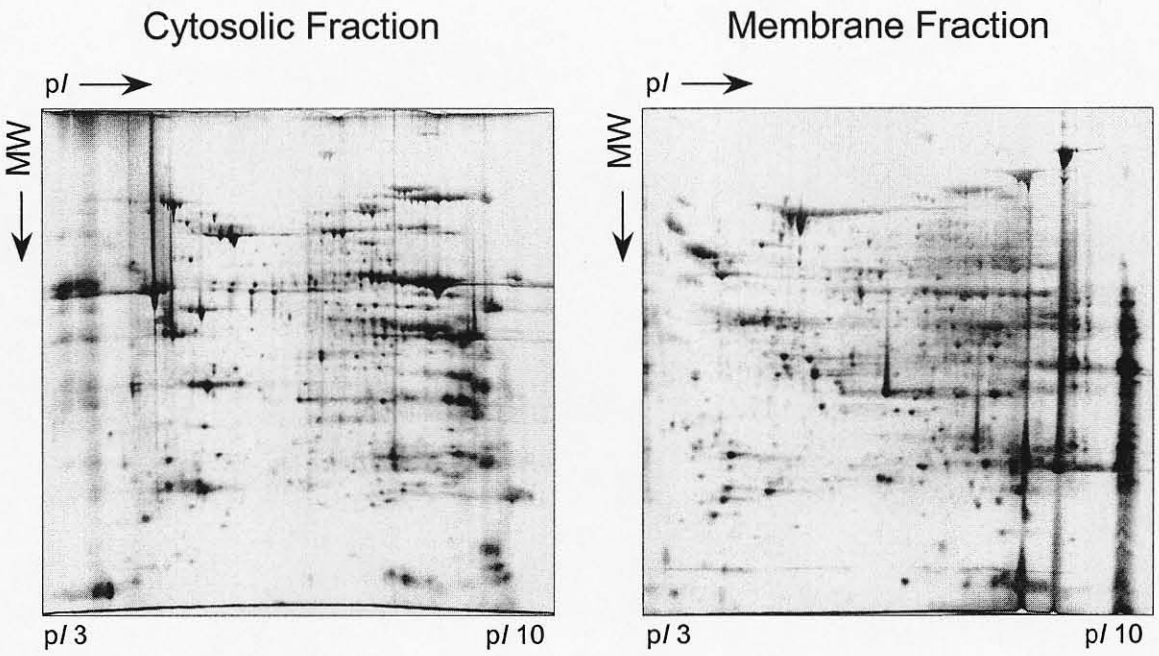
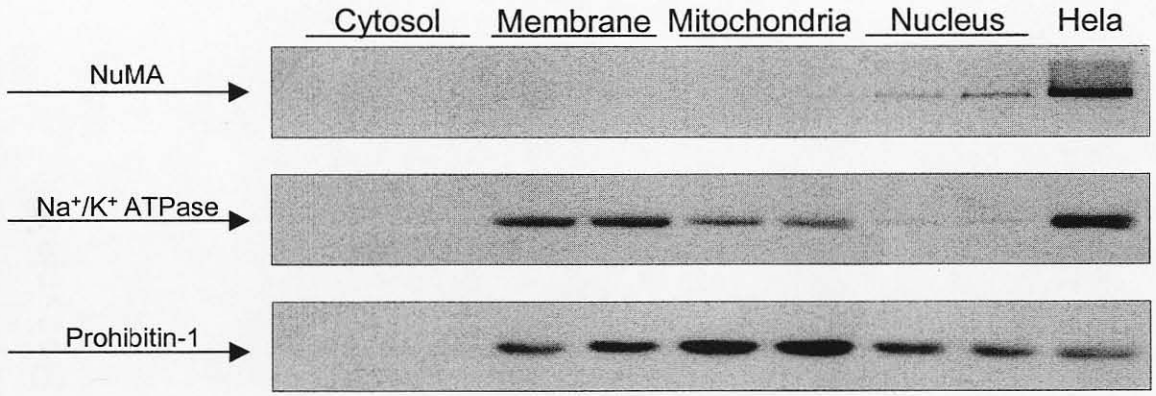

Cytosolic Fraction
Membrane Fraction
pI
MW
pI 3
pI 10
pI
MW
pI 3
pI 10

Figure 10. Immunoblotting analysis of known marker proteins in subcellular fractions. Subcellular-specific marker proteins were used to verify the separation procedure, such as nuclear-specific NuMA (upper panel); membrane-specific Na^+/K^+ ATPase (middle panel); and mitochondrial-specific Prohibitin-1 (lower panel). Cytosolic-specific lactate dehydrogenase (LDH) activity was also measured in each subcellular fraction (graph).


Cytosol
Membrane
Mitochondria
Nucleus
Hela
NuMA
Na+/K+ ATPase
Prohibitin-1

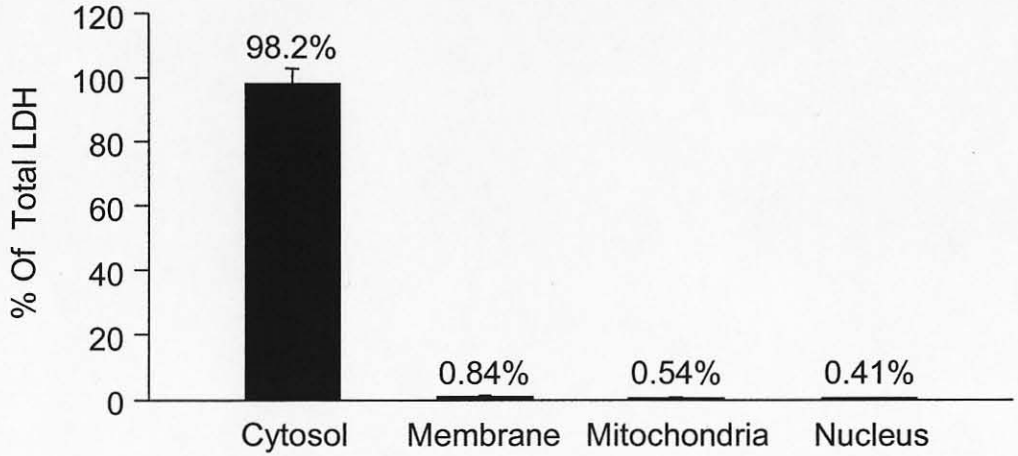

% Of Total LDH
120
100
80
60
40
20
0
98.2%
0.84%
0.54%
0.41%
Cytosol
Membrane
Mitochondria
Nucleus

Figure 11. Characterization of multiprotein PKCε signaling complexes-A. Protein from non-transgenic (NTG) and PKCε transgenic (TG) mouse hearts were immunoprecipitated (IP) with PKCε antibodies, separated by 1D SDS-PAGE, and immunoblotted (IB) with PKCε antibodies. This figure showed that PKCε could be efficiently immunoprecipitated from each subcellular fraction. Moreover, as expected that PKCε protein expression level in every compartment were higher in PKCε transgenic mice when compared with NTG.

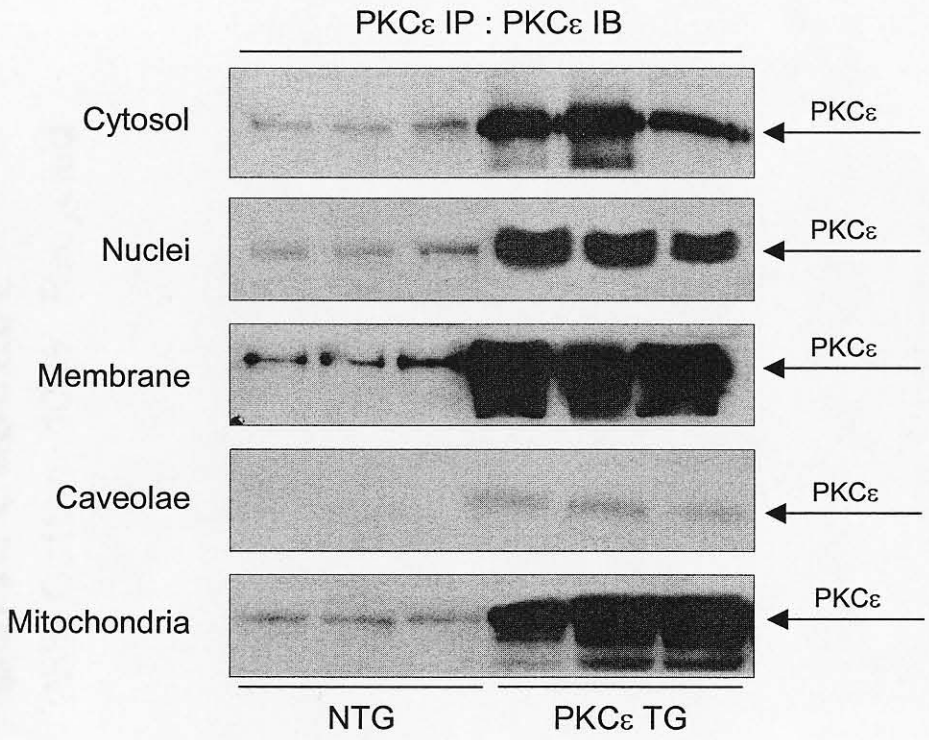

PKCε IP : PKCε IB
Cytosol
Nuclei
Membrane
Caveolae
Mitochondria
PKCε
PKCε
PKCε
PKCε
PKCε
NTG
PKCε TG

Figure 12. Characterization of multiprotein PKCε signaling complexes-B. Subcellular extracts were subjected to PKCε immunoprecipitation to isolate PKCε protein complexes. Then PKCε associated proteins were separated by 1D SDS-PAGE, and visualized by silver staining. PKCε associated with many proteins, and the patterns of polypeptides were significantly different in each subcellular fraction. The differences were including the number of proteins and protein molecular weight.

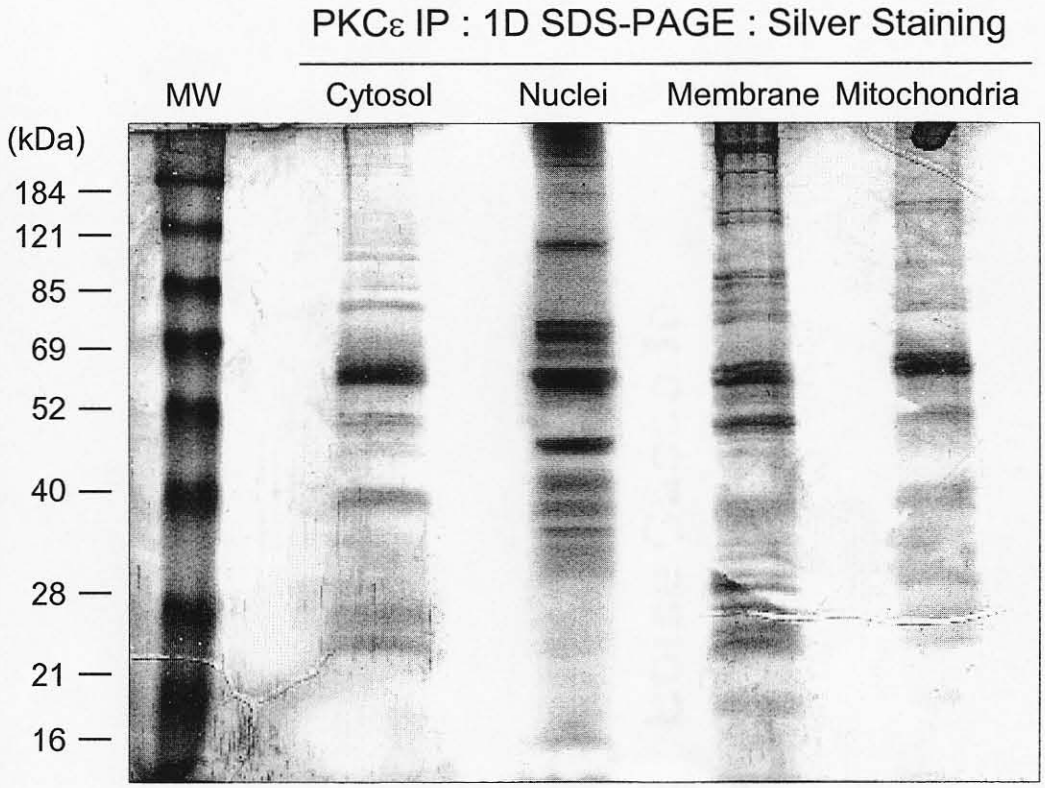
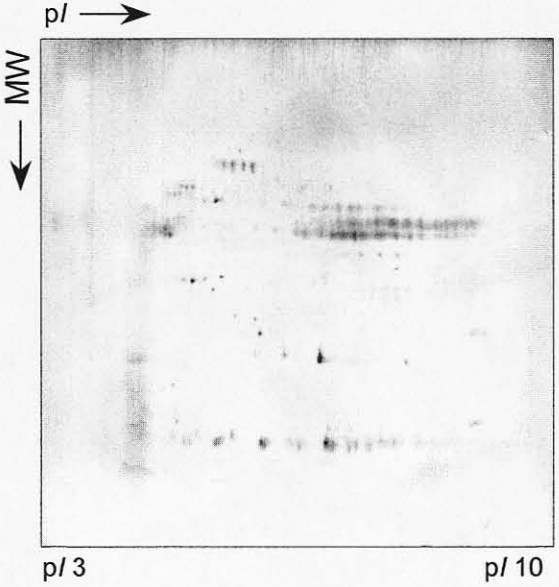

PKCε IP : 1D SDS-PAGE : Silver Staining
MW
Cytosol
Nuclei
Membrane
Mitochondria
(kDa)
184
121
85
69
52
40
28
21
16

Figure 13. Characterization of multiprotein PKCε signaling complexes-C. Subcellular extracts were subjected to PKCε immunoprecipitation to isolate PKCε protein complexes. Then PKCε associated proteins were separated by 2D SDS-PAGE, and visualized by silver staining.

PKCε IP : 2D SDS-PAGE : Silver Staining

PKCε signaling subproteome
Cytosolic Fraction



PKCε signaling subproteome
Nuclear Fraction

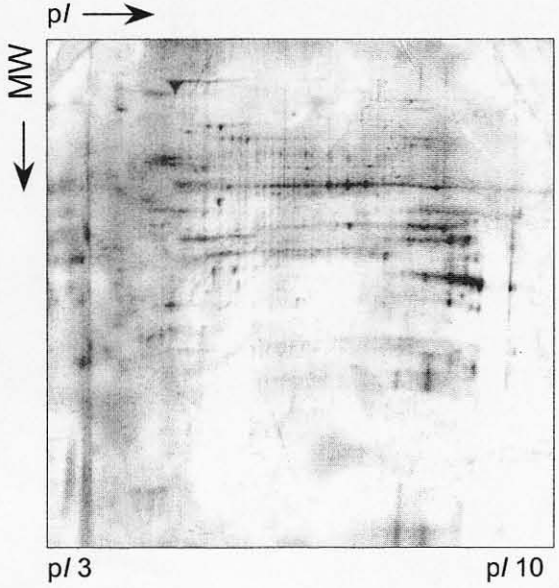
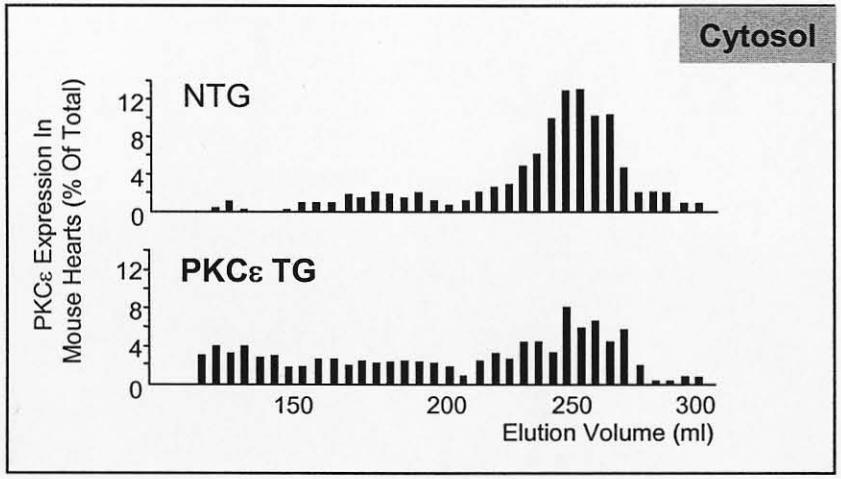
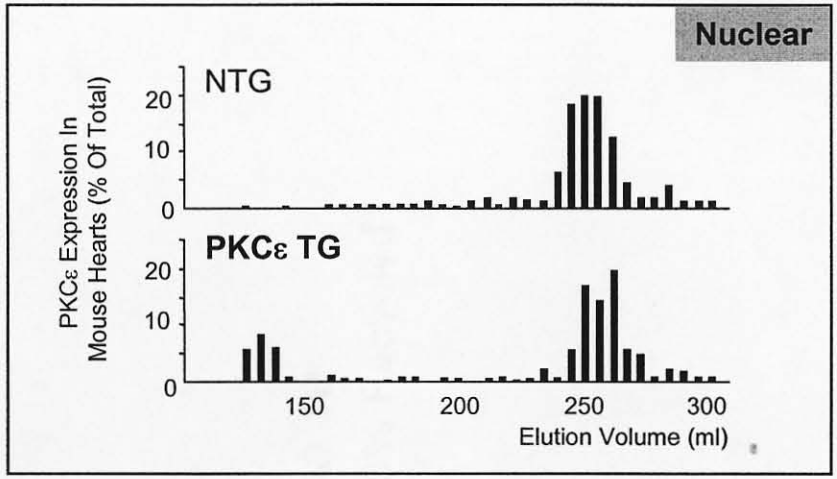
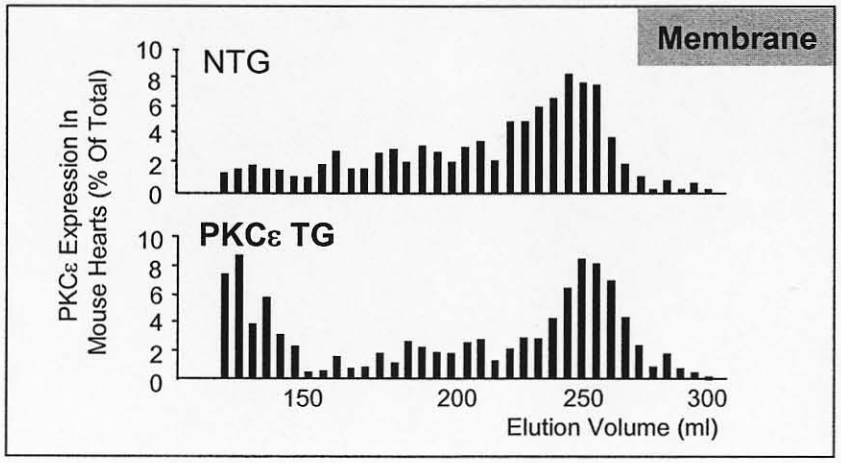


Figure 14. Demonstration of PKCϵ multiprotein signaling complexes by liquid chromatography. Subcellular protein lysates (~10mg) of both non-transgenic (NTG) and PKCε transgenic (TG) hearts were fractionated by gel filtration. Proteins from elution fractions were precipitated and PKCε signaling complexes visualized by immunoblotting. All elution fractions were normalized for PKCε expression using 100μg NTG heart lysates as internal control. Bar graph indicates the presence of PKCε multiprotein complexes. The elution profiles of PKCε complexes were different in different subcellular compartments. Compared with non-transgenic animals, the elution profiles of PKCε complexes were shifted toward higher PKCε complexes in PKCε-cardioprotective transgenic mouse hearts.


Cytosol
NTG
PKCε TG
PKCε Expression In Mouse Hearts (% Of Total)
12
8
4
0
150
200
250
300
Elution Volume (ml)


Nuclear
NTG
PKCε TG
PKCε Expression In Mouse Hearts (% Of Total)
20
10
0
150
200
250
300
Elution Volume (ml)


Membrane
NTG
PKCε TG
PKCε Expression In Mouse Hearts (% Of Total)
10
8
6
4
2
0
150
200
250
300
Elution Volume (ml)

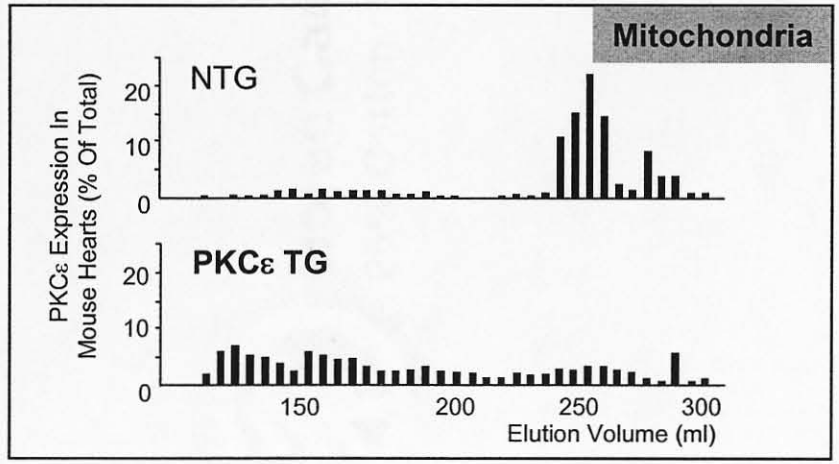

Mitochondria
NTG
PKCε TG
PKCε Expression In Mouse Hearts (% Of Total)
20
10
0
150
200
250
300
Elution Volume (ml)

Figure 15. Determination of the native size of multiprotein PKCε complexes.

(a) The 120th and 180th elution fractions from the gel filtration column were immunoprecipitated with PKCε antibodies. PKCε protein complexes were biotinylated with a trifunctional cross-linking agent (EZ-Link Sulfo-NHS-LC-Biotin [Pierce]). The biotinylated proteins were separated by 1D SDS-PAGE and visualized by immunoblotting using a streptavidin-HRP conjugate. (b) Native PAGE analysis of 180th elution fraction. Left column: various cellular multiprotein complexes visualized by native PAGE followed by silver staining. Right column: the PKCε protein complexes visualized by native PAGE followed by immunoblotting (IB) for PKCε (indicated by arrows). (c) Sedimentation sucrose gradient analysis of GST-PKCε-associated proteins. GST-PKCε fusion proteins were used for the affinity pull down PKCε complexes from cytosol. Subsequently, various sizes of GST-isolated protein mixtures were separated by sucrose gradient (10-30%). Upper panel: PKCε immunoblotting of the sucrose gradient fractions collected (1-to-18), arrows indicating the molecular weight range of PKCε multiprotein complexes (upper arrows at 232kDa and 669kDa, respectively). Lower panel: sucrose gradient fractions (1-to-18) were further separated by SDS PAGE and proteins visualized by silver staining.

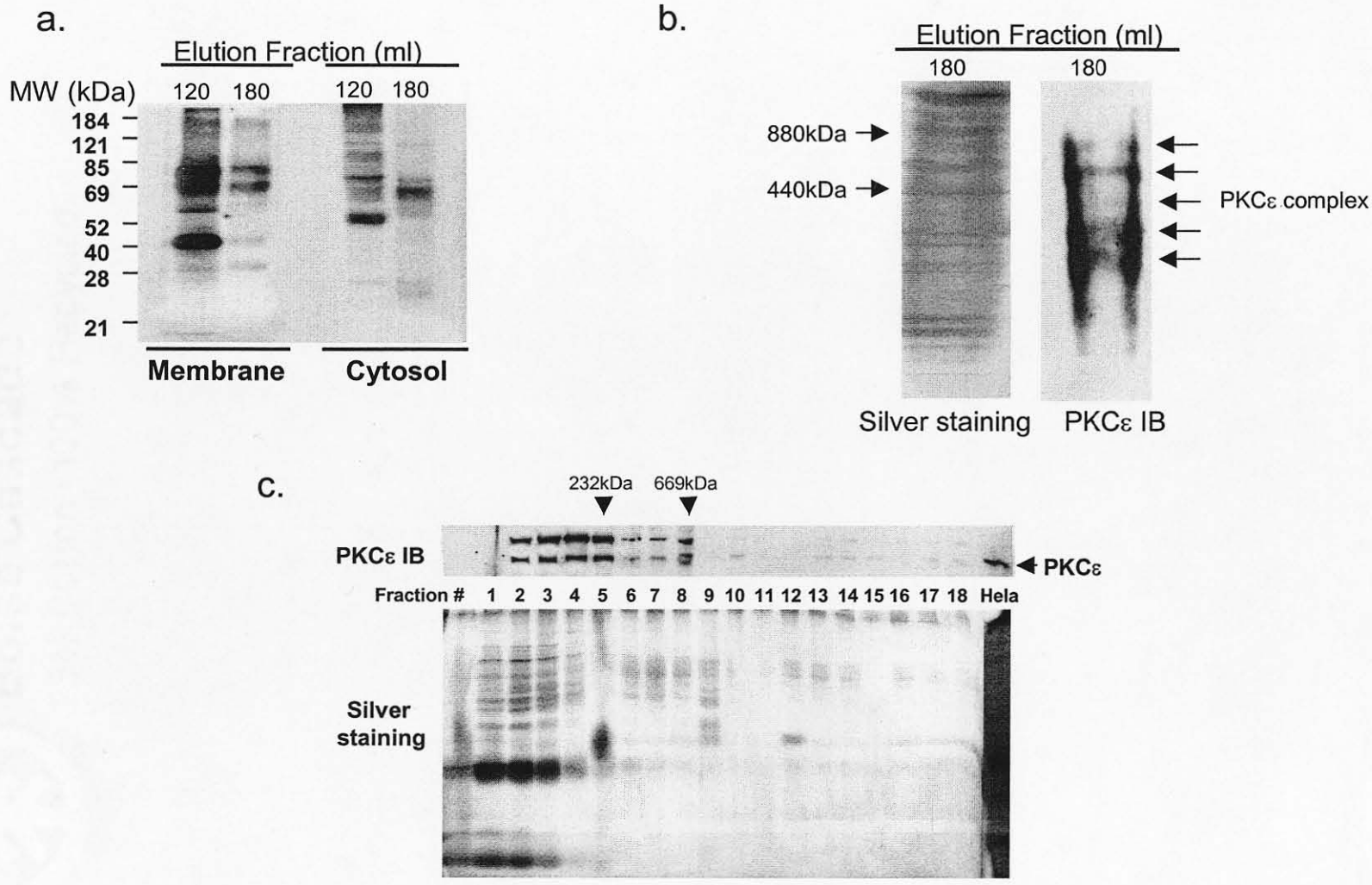

a.
Elution Fraction (ml)
MW (kDa)
120
180
120
180
184
121
85
69
52
40
28
21
Membrane
Cytosol
b.
Elution Fraction (ml)
180
180
880kDa
440kDa
PKCε complex
Silver staining
PKCε IB
c.
232kDa
669kDa
PKCε IB
PKCε
Fraction #
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Hela
Silver staining

Figure 16. Identification of the molecular components of the multiprotein PKCε complexes: examples of eNOS and p170 PI 3-Kinase.

Proteins from non-transgenic (NTG) and PKCε transgenic (TG) mouse hearts were separated into cytosolic, membrane, mitochondrial, nuclear and caveolae fractions. Then subcellular extracts were immunoprecipitated (IP) using PKCε antibodies, separated by 1D SDS-PAGE and immunoblotted (IB) with eNOS antibodies (left panels) and p170 PI 3-kinase antibodies (right panels).

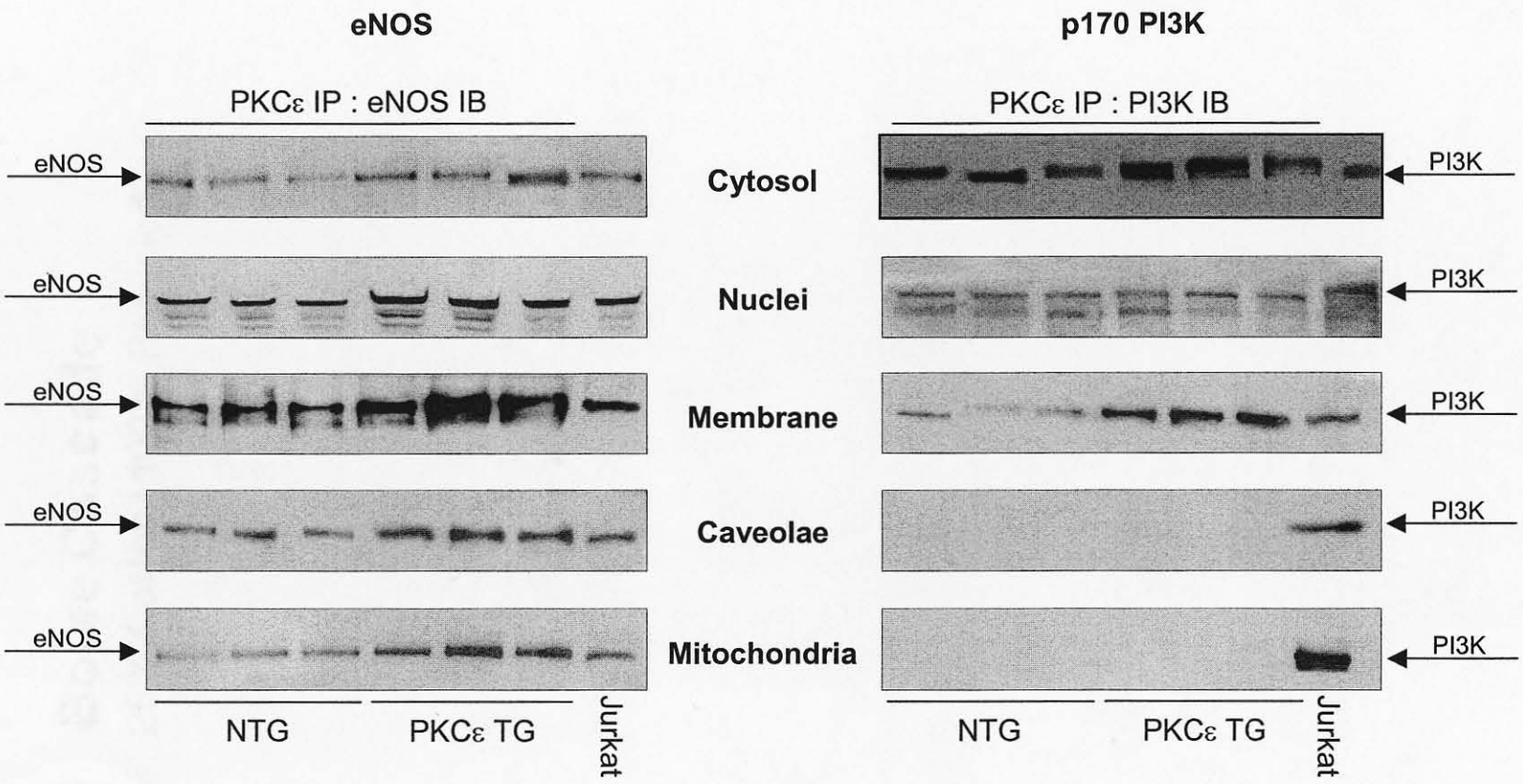

eNOS
p170 PI3K
PKCε IP : eNOS IB
PKCε IP : PI3K IB
eNOS
eNOS
eNOS
eNOS
eNOS
Cytosol
Nuclei
Membrane
Caveolae
Mitochondria
PI3K
PI3K
PI3K
PI3K
PI3K
NTG
PKCε TG
Jurkat
NTG
PKCε TG
Jurkat

Table 1 : PKCε signaling complex proteins in subcellular fractions

	M_r, (kDa)	pI	Known Function	Subcellular Fractionations				
				C	N	M	Mit	Cav
Structural proteins								
Troponin I	24	9.6	Contractile protein	+				
Troponin T	35	5.2	Contractile protein	+				
Desmin	53	5.2	Cytoskeletal protein	+		+		
RACK1	36	7.6	PKC binding protein		+	+		
RACK2	102	5.2	PKC binding protein	+	+	+	+	+
Villin	95	5.7	Cytoskeletal protein	+		+		
β'-Adaptin	106	5.0	Intracellular transport adaptor protein	+		+		+
AKAP79	79	5.1	Anchoring protein	+	+	+	+	
Vimentin	54	5.1	Intermediate filament	+	+	+		
Cadherin-2	129	4.9	Cell junction adhesions, Ca^{++} regulated	+	+	+	+	
Lamin C	65	6.4	Nuclear intermediate filament	+	+	+		
Oracle1/2	70/80	8.0	Cardiac development. LIM/PDZ domains	+	+	+	+	
Vinculin	117	5.7	Focal adhesion protein	+		+		
Glucose-regulated protein	75	5.1	Molecular chaperone	+		+		
Alpha-Actinin-2	104	5.4	Intermediate filament, Z band	+		+	+	
Lap2	75	8.6	Nuclear structural protein		+			
NMA	240	5.	Nuclear protein involved in mitosis	+	+			
GM130	130	5.0	Golgi matrix protein			+		
Prohibitin-1	30	5.6	Mitochondrial chaperone protein				+	
Caveolin-3	18	5.5	Cardiac caveolae structural protein					+
Signaling proteins								
PI 3-kinase	170	8.3	Phosphoinositide lipid kinase	+	+	+		
Pyk2	116	5.9	FAK tyrosine kinase family	+	+	+		
Lck	56	5.2	Src family tyrosine kinase	+	+	+	+	
Src	60	7.9	Src family tyrosine kinase	+	+	+	+	+
PKB/Akt	59	5.6	Stress-activated kinase	+	+	+	+	+
p38 MAPK	38	5.5	Stress-activated kinase	+	+	+	+	+
JNK1/2	46/54	7.6/6.2	Stress-activated kinase	+	+	+	+	+
ERK1/2	44/42	6.0/8.9	Receptor and stress-activated kinase	+	+	+	+	+
MEK1	45	6.2	Upstream activator of ERK	+			+	
Ras	21	6.2	GTPase that activates MAPK pathways	+	+	+	+	+
Connexin-43	43	9.0	Cardiac gap junction protein	+		+		
PDK1	63	6.7	Ser/Thr kinase	+	+	+	+	
PP2A	36	5.3	Ser/Thr kinase phosphatase	+		+	+	
PP1	38	5.9	Ser/Thr kinase phosphatase	+	+	+		

PI-3-kinase	85	6.0	Phosphoinositide lipid kinase		+	+	+	+
BMX	80	8.6	Tec family tyrosine kinase		+	+	+	
Stress-activated proteins								
iNOS	130	7.8	Inducible nitric oxide synthase	+	+	+	+	+
eNOS	140	6.6	Constitutive nitric oxide synthase	+	+	+	+	+
COX-2	70	7.0	Prostaglandin H synthase	+	+	+	+	
Heme-oxygenase 1	32	6.1	Stress-activated protein	+	+	+		
Aldose reductase	36	6.7	Osmotic stress-activated protein	+	+		+	
Heat shock protein 70	70	5.5	Molecular chaperone	+	+	+		+
Heat shock protein 27	25	6.4	Molecular chaperone	+	+	+		
αB-cystallin	20	7.3	Molecular chaperone	+	+	+		
Heat shock protein 60	61	6.3	Molecular chaperone	+			+	
Metabolism-related proteins								
ANT1/2	33	9.7	ATP/ADP exchanger	+			+	
Creatine kinase	43	6.6	Reversibly phosphorylates creatine, energy storage	+				
GAPDH	36	8.4	Glycolysis	+				
Isocitrate DH	38	6.0	Citric acid cycle	+	+		+	
VDAC	31	8.6	Mitochondrial pore component (ion transport)	+			+	
Transcription- & Translation-Related Proteins								
Histone H4	14	11.3	DNA binding to facilitate chromatin structure	+	+			
hnRNP H1	49	5.9	mRNA processing and regulation			+		
hnRNP K	51	5.3	mRNA processing and regulation	+	+			
Ribonucleoprotein	37	6.4	MRNA processing and regulation, heat shock, splicing	+	+	+		
NFκB p65	65	5.5	Activated by stress and inflammation	+	+			
HIF-1α	100	5.2	Hypoxia-inducible transcription factor	+	+			
c-Fos	55	4.8	Proto-oncogene, part of AP1 complex		+			
c-Jun	36	8.9	Proto-oncogene, part of AP1 complex		+			
CREB	43	5.4	Binds cAMP response element		+			
Elk	45	6.3	Activated by MAPK family kinases		+			
STAT1	87	5.4	Activated by JAK family kinases		+			
STAT3	88	5.9	Activated by JAK family kinases	+	+			
c-Myc	50	5.5	Proto-oncogene	+	+			
hnRNP a2/b1	37	9.0	MRNA processing and regulation		+			

All pI values were estimated using the ExPASy compute pI/MW tool available on the web.

C=Cytosol; N=Nuclei,; M=Membrane; Mit=Mitochondria; Cav=Caveolae.

Table 2 : Altered protein expression in PKCε signaling complex proteins in subcellular fractions

	Subcellular Fractionations				
	Cytosol	**Nuclear**	**Membrane**	**Mitochondria**	**Caveolae**
Structural proteins					
RACK2	++		++		
Villin			++		
AKAP79		++			
Oracle1/2		++			
GM130			++		
Caveolin-3					++
Signaling proteins					
PI 3-kinase (85kDa)					++
Pyk2 kinase	++				
Lck	++		++	++	
Src			++		
PKB/Akt		++	++		
p38 MAPK				++	
JNK1/2		++		++	
ERK1/2			++	++	
Ras	++	+++	+++		
PDK1			++	++	
BMX		++	++	++	
PI 3-kinase (170kDa)	++		++		
Stress-activated proteins					
eNOS	++	++	++	++	++
Aldose reductase	++			++	
Heat shock protein 27	++	++			
αB-cystallin			++		
Transcription- & Translation-Related Proteins					
NFκB p65		++			
HIF-1α		++			
c-Jun		++			
STAT3		++			

The "++" signs indicate increased expression of proteins in various subcellular compartments in cardiac protected PKCε transgenic mice. "++" reflects a change of 1-2 fold, "+++" a change of 2-3 fold.

CHAPTER VI

Functional proteomic analysis of myocardial PKCε-Akt-eNOS signaling modules during cardioprotection

Abstract

Having established in the studies described in the foregoing Chapter that PKCε complexes are differentially regulated in a subcellular compartment specific fashion, we next wanted to determine the manner in which individual signal transduction modules are formed to transducer signaling within these complexes.

Among the 93 different PKCε-associated proteins that we identified to date, the protective kinase Akt and stress-activated protein endothelial nitric oxide synthase (eNOS) are of importance due to their independent abilities to promote cell survival and prevent cell death. Their co-localization within the PKCε protein complex suggests that PKCε, Akt and eNOS may form signaling modules for the regulation of nitric oxide (NO) production and hence the manifestation of the cardioprotective phenotype. Accordingly, using functional proteomic tools, we found that: (1) PKCε, Akt and eNOS interact and form signaling modules both *in vitro* and *in vivo*; activation of either PKCε or Akt enhances the formation of PKCε-Akt-eNOS signaling modules; moreover, the formation of these signaling modules is dynamically regulated during cardioprotection; (2) PKCε directly phosphorylates and enhances activation of Akt *in vitro,* and PKCε transgenesis increases both phosphorylation and activation of Akt *in vivo*; (3) PKCε can also directly phosphorylate eNOS *in vitro*, and this phosphorylation enhances eNOS activity. Furthermore, overexpression of PKCε *in vivo* increased phosphorylation of eNOS at Ser1177, representing increased eNOS activity. This study characterizes, for the first time, functional as well as physical

coupling of PKCε, Akt, and eNOS in the heart, and provides novel information regarding the formation and modulation of PKCε-Akt-eNOS signaling modules during cardioprotection.

Introduction

The proteome is proposed to be composed of multiple integrated functional subproteomes, each of which is organized to facilitate a unique function. Moreover, specific signaling modules are formed within these subproteomes in response to different stimuli (82, 99, 170). In particular, our laboratory has focused on the PKCε subproteome, and the modules present within. PKCε plays a critical role in the genesis of cardioprotection against myocardial ischemic injury (49, 75, 140, 147), and cardiac-specific expression of PKCε is cardioprotective (142). Recent studies in our laboratory have found that PKCε forms multimeric signaling complexes with at least 93 proteins, including structural proteins, stress-activated proteins, proteins associated with transcription and translation, proteins involved in cellular metabolism, and various kinases and phosphatases (52, 142, 160, 172). Furthermore, the assembly of these multiprotein PKCε complexes is dynamically modulated in PKCε-transgenic mouse hearts, which serves as important evidence for the role of these complexes in regulating the cardioprotective phenotype. Among the 93 known protein partners, Akt and eNOS are of great interest as they are well established to independently promote cell survival and prevent cell death in response to various forms of stress (16, 42, 63, 182).

Previous studies in non-cardiac cells demonstrate physical and functional interactions among PKC, Akt and eNOS. Akt can directly interact and phosphorylate eNOS on Ser1177 (63). Akt co-immunoprecipitates with PKCζ or PKCι (48, 119), and the PH domain of Akt has been implicated as the binding side for PKCζ to Akt1 and Akt3 (110). Li et al show that overexpression of PKCα stimulates Akt activity and suppresses apoptosis induced by interleukin 3 withdrawals in myeloid progenitor cells (114). In contrast, Wen and colleagues (176) report that in A549 and HEK293 cells, the phosphatidylinositol 3-kinase/Akt signaling pathway is negatively regulated by PKC. Moreover, it is reported that activation of PKC inhibited eNOS activity by attenuating eNOS

phosphorylation on Ser1177 and increasing phosphorylation of Thr495 in endothelial cells (123, 124). However, despite this information nothing is known regarding the formation and regulation of PKCε-Akt-eNOS signaling modules in myocytes, especially during cardioprotection. Therefore, a comprehensive investigation needs to be implemented to draw a clearer picture of the temporal and spatial relationship among PKCε, Akt and eNOS in the regulation of NO production and the genesis of cardioprotection.

Accordingly, we hypothesized that PKCε, Akt and eNOS form a signaling module within the mouse heart, the assembly and activation of which contributes to the genesis of cardioprotection. By applying functional proteomic tools, we examined the PKCε-Akt-eNOS signaling modules *in vitro* and *in vivo* in two major aspects: molecular architecture, as defined by protein-protein interactions; and module regulation, as defined by posttranslational modification and alteration of activity of each component of PKCε-Akt-eNOS signaling. Moreover, using mice with cardiac-specific expression of active PKCε, we also assessed how these signaling modules are regulated during cardioprotection.

Results

PKCε can directly interact with Akt and eNOS *in vitro*

To examine the ability of PKCε to directly interact with Akt or eNOS, GST-PKCε fusion proteins were incubated with recombinant Akt or eNOS, followed by SDS-PAGE and Western blotting for Akt or eNOS. Figure 17 demonstrates that PKCε directly interacts with Akt *in vitro*. Moreover, we also found that GST-PKCε preferentially interacts with recombinant active Akt that is expressed by baculovirus in Sf21 insect cells and activated with GST-MAPKAPK2 and PDK1, as compared to a much weaker interaction with inactive Akt from insect cells without treatment. Furthermore, we assessed the domain-specificity of the interaction between Akt and PKCε by incubating GST-PKCε with recombinant Akt protein with or without PH domain. We found that the interaction between GST-PKCε and mutant, PH domain deficient, Akt was significantly attenuated, suggesting that this region of Akt is responsible for its interaction with PKCε (Figure 17a and b). Taken together, our data indicated that PKCε directly interacts with Akt primarily through binding Akt's PH domain. Using a similar approach, we also found that GST-PKCε could also physically directly interact with eNOS, as shown in Figure 17c.

Since PKCε can directly interact with Akt and eNOS *in vitro* in a binary sense, we then assessed the ability of these molecules to enhance their association with each other beyond that seen in the foregoing pair-wise experiments. Recombinant Akt and eNOS were incubated with or without PKCε followed by eNOS immunoprecipitation and Akt immunoblotting. We found that recruitment of PKCε increased the binding affinity of eNOS with Akt (Figure 18a left). To examine the effect of PKCε activity on this interaction, recombinant PKCε was next incubated with Akt and eNOS in the presence or absence of potent PKC activators phorbol ester (PMA) and phosphatidylserine (PS) followed by immunoprecipitation of PKCε and blotting for Akt or eNOS.

Figure 18a (right) showed that although PKCε formed protein complexes with Akt and eNOS in the absence of PKC activators, PMA/PS-activated PKCε exhibited a much high affinity for Akt and eNOS, indicating that activation of PKCε enhanced its interaction with both Akt and eNOS. In addition, we tested the effect of Akt on the interaction of PKCε and eNOS by incubating PKCε and eNOS with active or inactive Akt recombinant proteins. Similar to the results obtained for PKCε, activation of Akt was found to enhance its interaction with PKCε and eNOS, and its ability to facilitate interactions between PKCε and eNOS (Figure 18b).

PKCε interacts with Akt and eNOS *in vivo*

Previous studies have shown that Akt and eNOS are present in cardiac PKCε-signaling complexes, as assessed by both GST-PKCε affinity pull down assay and PKCε immunoprecipitation (52, 142), indicating that PKCε can interact with Akt and eNOS *in vivo*. In the present study, using PKCε immunoprecipitation, we found that although all three isoforms of Akt are present in the mouse heart, only Akt1 and Akt2 interact with PKCε (Data not shown).

To assess the formation of PKCε-Akt-eNOS signaling modules *in vivo*, mouse hearts were homogenized and subjected to gel filtration chromatography using a Sephacryl S400 column. This process separates intact myocardial protein complexes on the basis of their physical size. Individual fractions from liquid chromatography were next immunoprecipitated with PKCε antibodies. The PKCε immuno-complexes were then separated by SDS-PAGE and blotted for PKCε, Akt and eNOS. Figure 19a shows the chromatographic profiles of PKCε-associated Akt and eNOS protein complexes in normal mouse hearts. We found that Akt and eNOS co-precipitated with PKCε in multiple fractions, indicating that PKCε, Akt and eNOS interacted with each other in a variety of different sized PKCε-containing complexes. Furthermore, compared with the profiles of non-transgenic mouse hearts, we found that the expression profiles of PKCε-Akt-eNOS signaling modules in PKCε-cardioprotective transgenic mice were shifted towards higher molecular weight complexes (Figure 19b), indicating the dynamic modulation of PKCε-Akt-eNOS signaling modules *in vivo* during cardioprotection.

PKCε can directly phosphorylate Akt and eNOS and enhance their activities *in vitro*.

Based on the above findings, we further investigated the effect of PKCε activation on the formation of PKCε-Akt-eNOS modules *in vitro*. First, we assessed whether PKCε could directly phosphorylate Akt and/or eNOS *in vitro*. Recombinant Akt or eNOS were incubated with recombinant PKCε in the presence of the PKC activators, phorbol ester (PMA) and phosphatidylserine (PS), and radiolabeled P^{32}-γ-ATP. After SDS-PAGE separation, phosphorylation was visualized by autoradiography. We found that PKCε could directly phosphorylate both Akt and eNOS (Figure 20a).

Next, we further tested the effects of these PKCε-induced phosphorylation modifications on the activation status of Akt and eNOS. After incubating recombinant inactive Akt with PKCε, Akt activity was assessed by its ability to phosphorylate GSK-3β. We found that Akt activation was dramatically increased with the PKCε treatment (Figure 20b) as compared with inactive Akt alone, indicating that activated PKCε dramatically enhances Akt activity *in vitro*. Similarly, we found that PKCε treatment significantly enhanced eNOS activity, as measured by nitrate/nitrite production, (178.8±11%, $P<0.05$ vs eNOS only) when compared with non-treatment (Figure 20b).

Studies by other investigators indicate that Akt is activated by phosphorylation of Thr308 within its activation loop which serves to stabilize the molecule in its active confirmation. Subsequently, phosphorylation of serine 473 (Ser473) at the C-terminus is essential for full activation of Akt. While it is well known that PDK1 is responsible for Thr308 phosphorylation, the upstream kinases that catalyze the phosphorylation of Ser473 is unclear. Because our initial studies demonstrated signal transduction between PKCε and Akt, we next tested whether PKCε could be a putative PDK2 that targets Ser473 by using the site-specific phospho-antibody of Akt after the PKCε treatment. Figure 21 shows that addition of recombinant PKCε significantly increased Ser473 phosphorylation on Akt as compared with that of inactive Akt alone, indicating that *in vitro*, PKCε directly phosphorylates Akt on Ser473.

PKCε transgenesis enhances phosphorylation and activation of Akt and eNOS *in vivo*

In addition to the *in vitro* phosphorylation experiments, we also examined the phosphorylation of Akt and eNOS *in vivo*. PKCε–cardioprotected transgenic mice hearts were used to test the effect of activation of PKCε on Akt by examining the two conserved phosphorylation sites of Akt. Using the site-specific phospho-antibodies of Akt, we found that phosphorylation of Akt on both activation sites Thr308 (336.1±15.1% vs NTG) and Ser473 (181.3±18.2% vs NTG), was significantly increased in PKCε transgenic mice when compared with non-transgenic animals. These post-translational modification changes occurred in the absence of a total change in Akt protein expression (Figure 22a). Interestingly, total cardiac cellular Akt activity was increased in PKCε cardioprotected mice (Figure 22b top panel). Moreover, when we examined PKCε-associated Akt activity (PKCε complexes were isolated by immunoprecipitation and Akt kinase activity assays performed), we found a surprising augmentation of Akt activity, considerably greater than that seen in the total lysate pool, when Akt was associated with PKCε (Figure 22b lower panel). These data support the concept that module formation between PKCε and Akt accomplishes signal transduction by post-translationally modifying Akt and increasing its activity on downstream substrates.

It is well documented that once Akt is activated, it can phosphorylate eNOS at serine 1177(Ser1177) resulting in activation of eNOS and the production of nitric oxide. Therefore, we examined the phosphorylation of eNOS *in vivo* in the PKCε transgenic mice hearts. Phosphorylation of eNOS on Ser1177 (407.8±43.7% vs NTG) was enhanced in the PKCε transgenic mice (Figure 23), indicating that overexpression of PKCε increased eNOS activity *in vivo*. Meanwhile, total eNOS protein expression was also slightly increased (140.9±7.2% vs NTG) in PKCε transgenic mice.

Discussion

Recent studies suggest that cellular biological functions, such as signal transduction, are accomplished by multi-protein complexes, which contain within them modules that are composed of several species of interacting molecules. Modules may have discrete functions engendered from the interactions among their individual components. Understanding how these molecules interact and regulate each other is critical in order to comprehend the specific biological function of such modules. Accordingly, in this study, we hypothesized that PKCε forms functional signaling modules with Akt and eNOS, and that the assembly and activation of these modules contributes to the genesis of cardioprotection. Using functional proteomic approaches, we tested the hypothesis in two different aspects: molecular architecture and module regulation. There are several important findings in this investigation. First, PKCε interacts with Akt and eNOS both *in vitro* and *in vivo*, and these interactions are promoted by enhanced activation of PKCε. Second, PKCε forms various sizes of signaling modules with Akt and eNOS, which are dynamically regulated in PKCε transgenic mice during cardioprotection. Third, PKCε directly phosphorylates Akt and eNOS, resulting in increased Akt and eNOS activation *in vitro*, whereas *in vivo*, overexpression of PKCε increases both phosphorylation and activation of Akt and eNOS, especially PKCε-associated Akt activation. This study characterizes, for the first time, physical and functional coupling of PKCε, Akt, and eNOS in the mouse heart, and provides novel information regarding the formation and modulation of PKCε-Akt-eNOS signaling modules during cardioprotection.

Spatial distribution of PKCε-Akt-eNOS signaling modules

In addition to the molecular architecture of signaling modules and their regulation, the spatial distribution of a module within the cell is another important aspect to understand the

function of these signaling units. Using classical immunoprecipitation and immunoblotting techniques, our previous studies already determined the subcellular distributions of PKCε-Akt-eNOS signaling modules both in normal and cardioprotective hearts. We found that, accompanying the increased protein expression of PKCε in multiple fractions (cytosol, membrane, mitochondria, nuclear, and caveolae), protein expression of eNOS in PKCε complexes was also increased in all five subcellular fractions. In contrast, Akt expression was only found to be increased in PKCε-associated *membrane* and *nuclear* fractions in cardioprotected mouse hearts. Therefore, although PKCε-Akt-eNOS modules were found to be existent in multiple fractions, increased formation of this module occurred only in membrane and nuclear fractions during cardioprotection, implicating subcellular compartment-specific functions of PKCε-Akt-eNOS modules in normal and protected hearts. For example, many studies have demonstrated that translocation to the membrane is an important regulatory action for activated PKCε and Akt. Moreover, eNOS is largely membrane-associated as a result of the N-terminal myristoylation and palmitoylation of this molecule (30). Thus, we expected to see the increased formation of PKCε-Akt-eNOS signaling modules in membrane fractions during cardioprotection. Increased formation of PKCε-Akt-eNOS signlaing modules in nuclear fractions in PKCε transgenic mice may modify activation of transcription factors or other targets within nucleus fraction and thereby regulate cardioprotective gene/protein synthesis.

PKCε forms different sizes of signaling modules with Akt and eNOS

Revealing the existence of intact, non-denatured, multiprotein PKCε-Akt-eNOS modules is an important step to confirm our hypothesis regarding the involvement of these modules in signal transduction. Using biochemical methods of gel filtration, PKCε immunoprecipitation, we have delineated the physical characteristics of PKCε-Akt-eNOS signaling modules. Our data clearly show that PKCε co-exists with Akt and eNOS in protein complexes of varying sizes in the mouse heart. Moreover, during PKCε-induced cardioprotection, the profile of PKCε-Akt-eNOS modules is dynamically regulated. These data provide PKCε-Akt-eNOS modules shifting to larger

molecular weight complexes. These data provide a solid base for our future study to explore the composition and interaction of each PKCε-Akt-eNOS signaling module.

To confirm the presence of PKCε-Akt-eNOS signaling modules *in vivo*, in addition to separation protein complexes following PKCε immunoprecipitation, we used GST-PKCε pull down assays to form new PKCε complexes, and then separated the complexes by deoxycholate-buffered native gel electrophoresis followed by Akt and eNOS immunoblotting. It is found that only a few of PKCε-Akt-eNOS modules had been formed in non-transgenic mouse hearts, whereas, a high molecular weight PKCε-Akt-eNOS signaling module was found in the PKCε-cardioprotected transgenic mice. The size of this macromolecular complex was estimated to be greater than 880kDa, as determined by comparison to protein markers (data not show). Interestingly, we found significantly fewer PKCε-Akt-eNOS modules via the native PAGE approach as compared with the results from the gel filtration technique. The difference between these results is most likely due to the fact that addition of exogenous PKCε will likely disrupt endogenous preexisting complexes while also inducing formation of other complexes. Thus, these techniques (immunoprecipitation and GST-based affinity pull-down) appear to be complementary, in that they identify non-overlapping pools of protein interactions.

Since PKCε interacts with Akt and eNOS to form varying sizes of complexes, how do these three molecules interact? Several investigators have shown that PH domain is the preferred interaction domain for PKC binding to Akt. In our study, we found that PKCε preferred to interact with Akt via its PH domain which was congruous with previous studies in non-cardiac cells (18). Moreover, we found that PH domain of Akt may not necessary for eNOS binding to Akt *in vitro* (data not shown). However, the detailed interactions among PKCε, Akt and eNOS are still under investigation.

PKCε is a positive regulator for the formation of PKCε-Akt-eNOS signaling modules.

Previous studies have focused on the *individual roles* of PKCε, Akt and eNOS as cardioprotective proteins. These studies include one in which cardiac-specific expression of constitutively active Akt was found to reduce infarct size and apoptosis after ischemia-reperfusion

injury (62). In another investigation, myocardial overexpression of eNOS was found to greatly stimulate cardiac nitric oxide production and to protect the heart against ischemia/reperfusion injury. Despite this, the effect of PKCε on the regulation of Akt and eNOS is controversial, and data regarding the association of these molecules is particularly lacking in cardiac cells. Amino acid sequence analyses reveal that both Akt and eNOS contain multiple potential PKC phosphorylation sites. In our study, we found that PKCε could directly phosphorylate Akt and eNOS *in vitro*, whereas, cardiac-specific overexpression of PKCε induces increased phosphorylation of both Akt and eNOS.

Previous studies have shown that overexpression of PKCα stimulated the activation of Akt. In contrast, several other investigators reported that in A549 and HEK293 cells, the phosphatidylinositol 3-kinase/Akt signaling pathway was negatively regulated by PKC (176). PMA-induced apoptosis was accompanied by inhibition of Akt activity (149). Similarly, PKC activation inhibited eNOS activity by attenuating eNOS phosphorylation on Ser1177 and increasing phosphorylation of Thr495 in cardiovascular endothelial cells (123, 124). Thus, it was unclear in what manner PKCε, Akt and eNOS might interact to regulate each other in the setting of cardioprotection. In the present study, cardiac PKCε was found to serve as a positive regulator of the formation and regulation of PKCε-Akt-eNOS signaling modules in mouse hearts. We found that activation of PKCε enhances the module formation, and the phosphorylation modification induced by PKCε may alter the conformation of both Akt and eNOS and thereby further enhance their activity both *in vitro* and *in vivo*. These data indicate that through the increased formation of PKCε-Akt-eNOS signaling modules, activation of PKCε may promote cell survival and stimulate nitric oxide synthesis, and therefore contribute to the genesis of cardioprotection against ischemic injury.

Regarding the finding that PKCε can directly phosphorylate eNOS and increase eNOS activity *in vitro*, the possibility exists that the purity of eNOS recombinant protein may influence the *in vitro* results. Nonetheless, the increased eNOS activity appear to be PKCε-dependent.

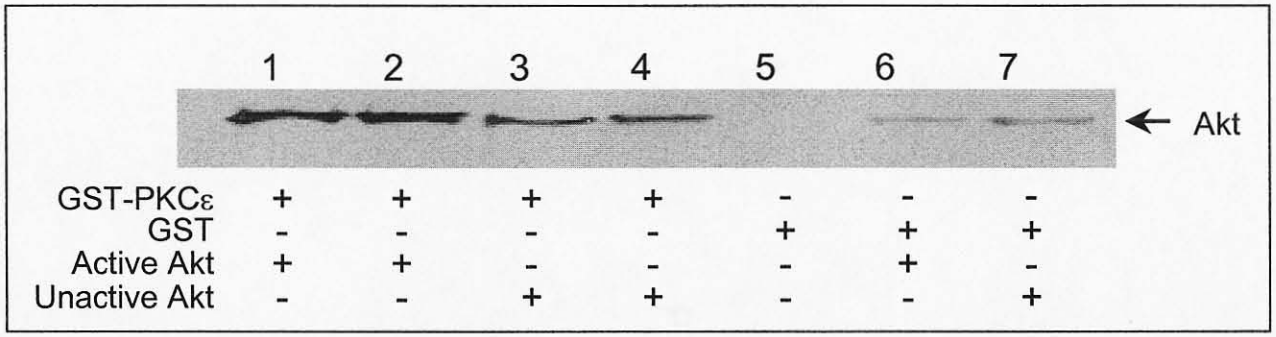
PKCε is a candidate for PDK2

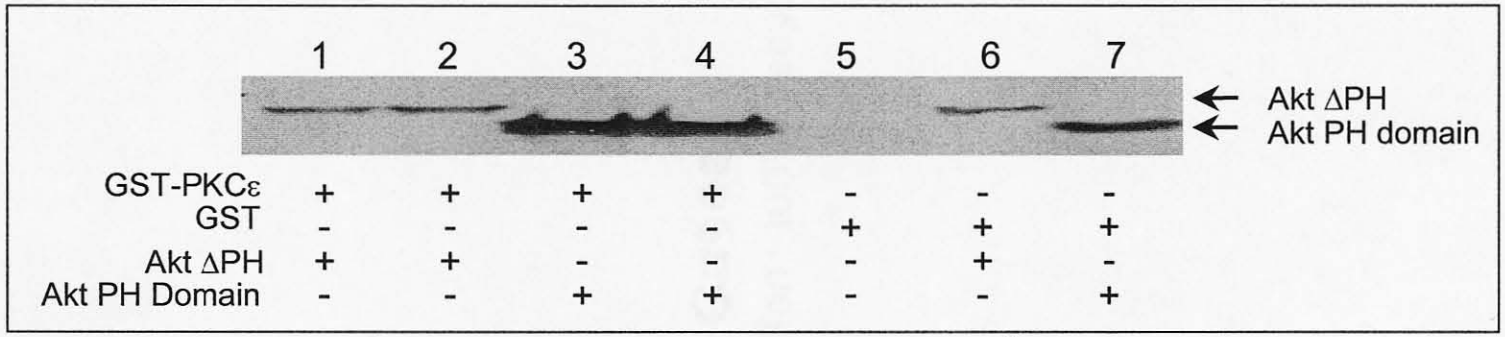
Akt has two conserved phosphorylation sites, Thr308, and Ser473, which are necessary for its maximum activation. Generally, the upstream kinase that fulfils the phosphorylation of Thr308 is PDK-1. But the upstream kinases that are responsible for the Ser473 phosphorylation is unknown, and has been tentatively named "PDK-2". Several candidates have been proposed, including PKCζ (34 ,190), a lipid-raft-associated kinase (84), or simply the autophosphorylation processes followed by the PDK-1 activity (1). In the present study, we tested the hypothesis that PKCε may exhibit PDK-2 activity by phosphorylating Akt Ser473. Interestingly, we found that PKCε did indeed phosphorylate Akt Ser473 *in vitro*, indicating PKCε maybe one of the candidates *in vivo* to target Akt and increase Akt activation by modifying Ser473 phosphorylation. Determination of the ability of PKCε to phosphorylate Akt on Ser473 *in vivo*, and the physiological importance of this post-translational modification is an extensive process that is beyond the scope of the current investigation.

In summary, this study successfully characterized myocardial PKCε-Akt-eNOS signaling modules, which can serve as a good model for future research addressing the signaling mechanisms underlying PKCε-mediated cardioprotection and other phenotypes. The present study provided new information about the molecular architecture of PKCε-Akt-eNOS signaling modules, the subcellular localization of these modules, and the dynamic modulation of these modules during the development of the cardioprotective phenotype. These studies are critical steps towards our goal of defining the cardiac proteome, the multiple integrated functional subproteomes that compose this proteome, and the specific signaling modules required for unique cellular functions.

Figure 17. PKCε directly interacts with Akt and eNOS *in vitro* GST-PKCε fusion proteins were incubated with recombinant Akt or eNOS, followed by SDS-PAGE and Western blotting for Akt or eNOS. a. GST-PKCε interacts with Akt *in vitro*, and preferentially interacts with recombinant active Akt when compared with recombinant inactive Akt; b. GST-PKCε had higher binding affinity with the Akt PH domain when compared with PH domain deleted Akt; c. GST-PKCε also physically interacts with eNOS *in vitro*.

a.


1 2 3 4 5 6 7
Akt
GST-PKCε + + + + - - -
GST - - - - + + +
Active Akt + + - - - + -
Unactive Akt - - + + - - +


1 2 3 4 5 6 7
Akt ΔPH
Akt PH domain
GST-PKCε + + + + - - -
GST - - - - + + +
Akt ΔPH + + - - - + -
Akt PH Domain - - + + - - +

b.

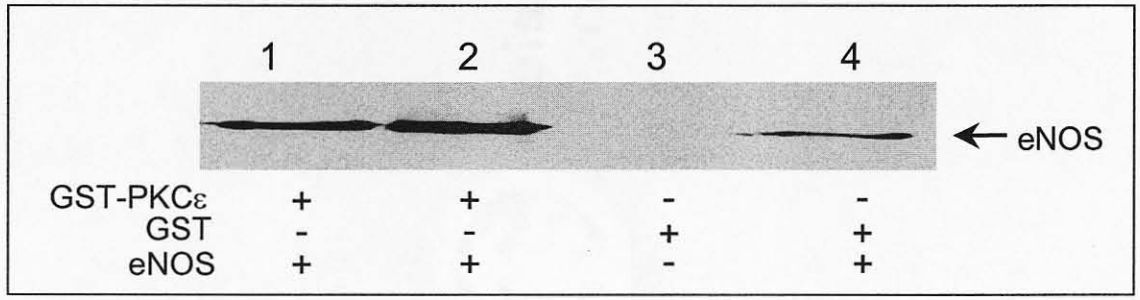
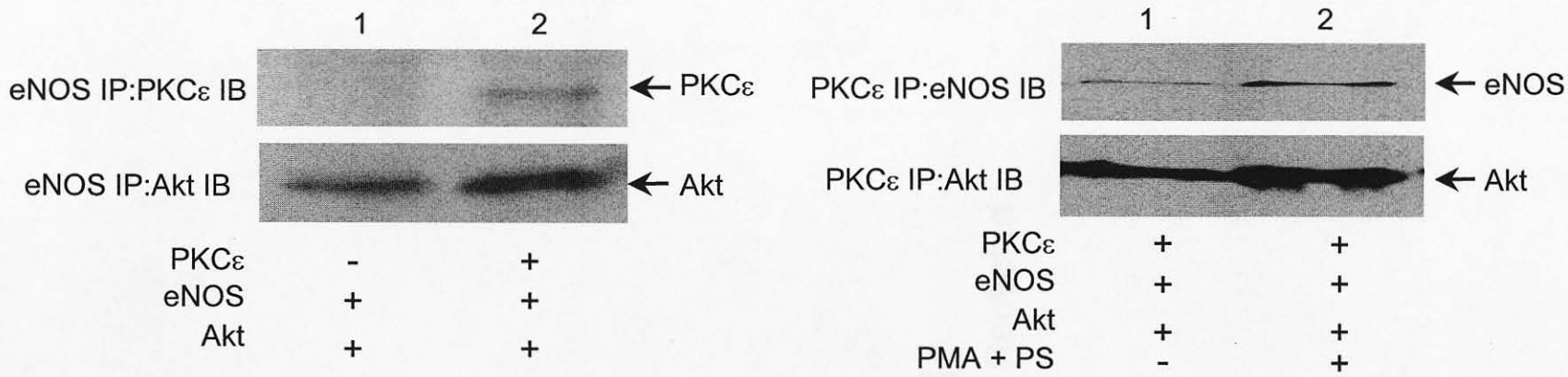

1 2 3 4
eNOS
GST-PKCε + + - -
GST - - + +
eNOS + + - +

Figure 18. Activation of both PKCε and Akt enhanced PKCε-Akt-eNOS module formation *in vitro*. a. Left, recombinant Akt and eNOS were incubated with or without PKCε followed by eNOS immunoprecipitation and Akt immunoblotting. Recruitment of PKCε increased the binding affinity of eNOS with Akt; right, recombinant PKCε was further incubated with Akt and eNOS plus or minus potent PKC activators phorbol ester (PMA) and phosphatidylserine (PS) followed by immunoprecipitation of PKCε and blotting for Akt or eNOS. PMA/PS-activated PKCε exhibited higher affinity for Akt and eNOS compared with that of PKCε without stimulation. b PKCε and eNOS with active or inactive Akt recombinant proteins. Activation of Akt also enhanced the interaction of PKCε with Akt and eNOS.

a.


1
2
eNOS IP:PKCε IB
PKCε
eNOS IP:Akt IB
Akt
PKCε - +
eNOS + +
Akt + +
1
2
PKCε IP:eNOS IB
eNOS
PKCε IP:Akt IB
Akt
PKCε + +
eNOS + +
Akt + +
PMA + PS - +

b.

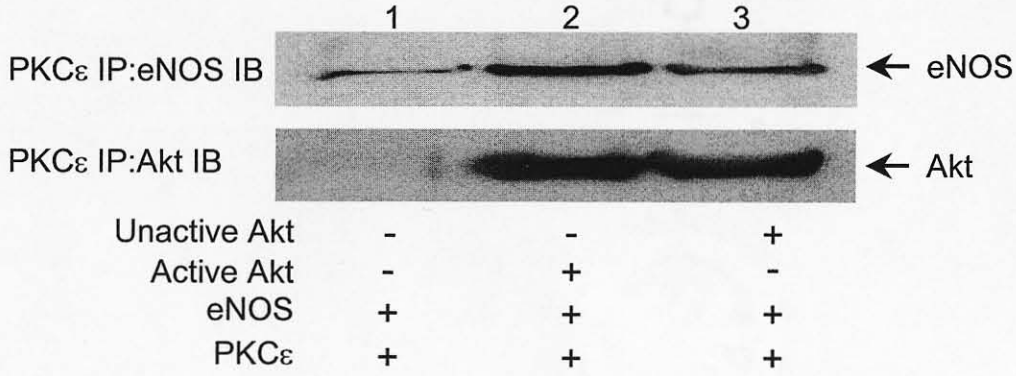
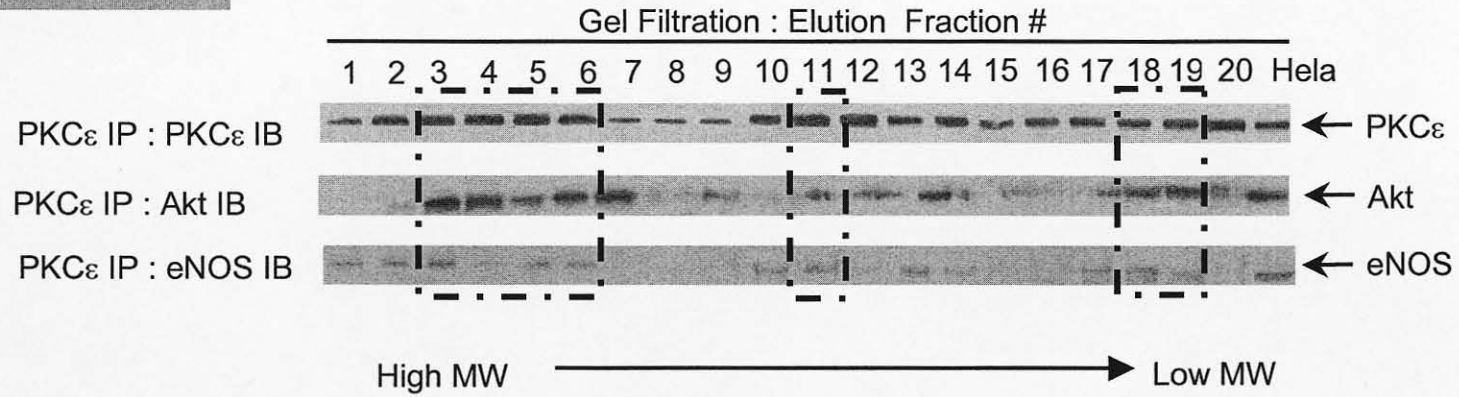

1
2
3
PKCε IP:eNOS IB
eNOS
PKCε IP:Akt IB
Akt
Unactive Akt - - +
Active Akt - + -
eNOS + + +
PKCε + + +

Figure 19. Formation of PKCε-Akt-eNOS signaling modules *in vivo*. Mouse hearts were homogenized and subjected to gel filtration chromatography using a Sephacryl S400 column followed by immunoprecipitation with PKCε antibodies and immunoblotting for PKCε, Akt and eNOS. a. The chromatographic profiles of PKCε-associated Akt and eNOS protein complexes in the normal mouse hearts; b the chromatographic profiles of PKCε-associated Akt and eNOS protein complexes in the PKCε-transgenic mouse hearts. Akt and eNOS co-precipitated with PKCε in multiple fractions to form a variety of different sized PKCε complexes. Compared with the profiles of non-transgenic mouse hearts, the expression profiles of PKCε-Akt-eNOS signaling modules in PKCε-cardioprotective transgenic mice were shifted towards higher molecular weight complexes.

a. NTG mouse hearts



b. PKCε TG mouse hearts

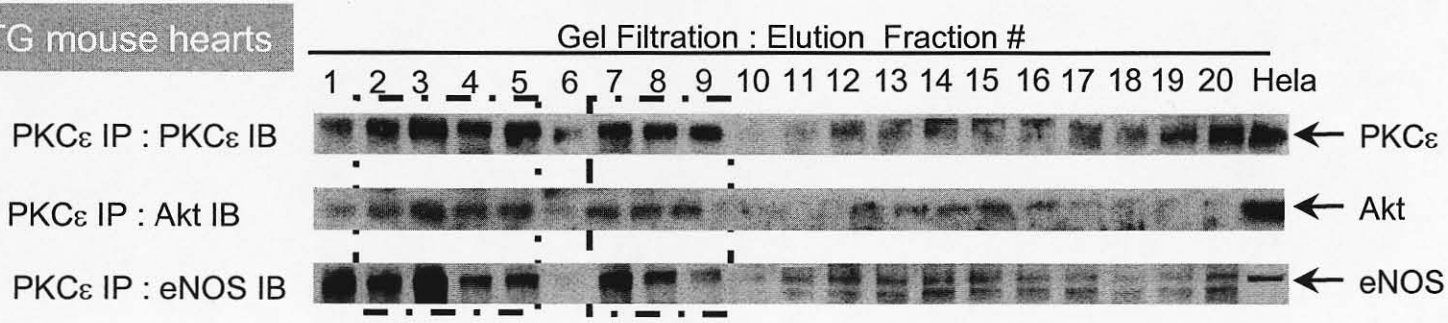
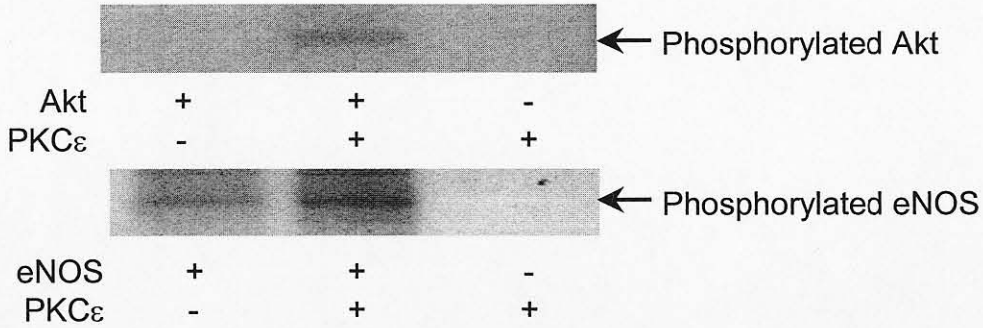


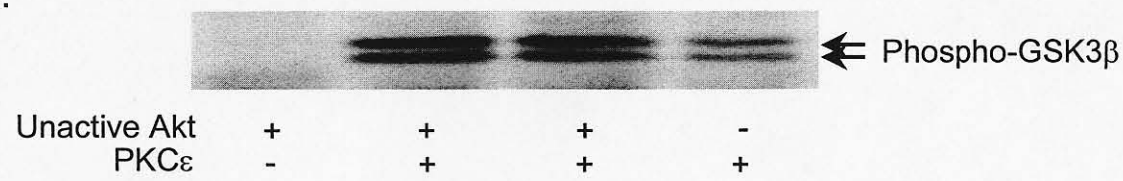
Figure 20. PKCε phosphorylates and enhances activation of Akt and eNOS *in vitro*.

a. Recombinant Akt or eNOS incubated with recombinant PKCε in the presence of the PKC activators phorbol ester (PMA) and phosphatidylserine (PS) and P^{32}-γ-ATP. After SDS-PAGE separation, phosphorylation was visualized by autoradiography; b. After incubating recombinant inactive Akt or eNOS with PKCε, Akt and eNOS activity were assessed by its ability to phosphorylate GSK-3β or produce nitrate/nitrite, respectively.

a. Akt and eNOS phosphorylation:



b. Akt *In vitro* activity:



eNOS *In vitro* activity:

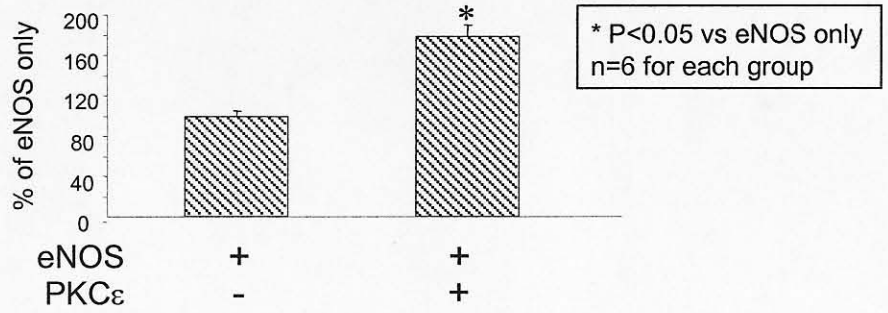


Figure 21. PKCε directly phosphorylated Akt at its Ser473 site *in vitro*. After incubating PKCε with recombinant Akt, Ser473 phosphorylation was detected by site-specific antibody phospho-Akt-Ser^{473}. Addition of recombinant PKCε significantly increased Ser473 phosphorylation on Akt as compared with that of inactive Akt alone.

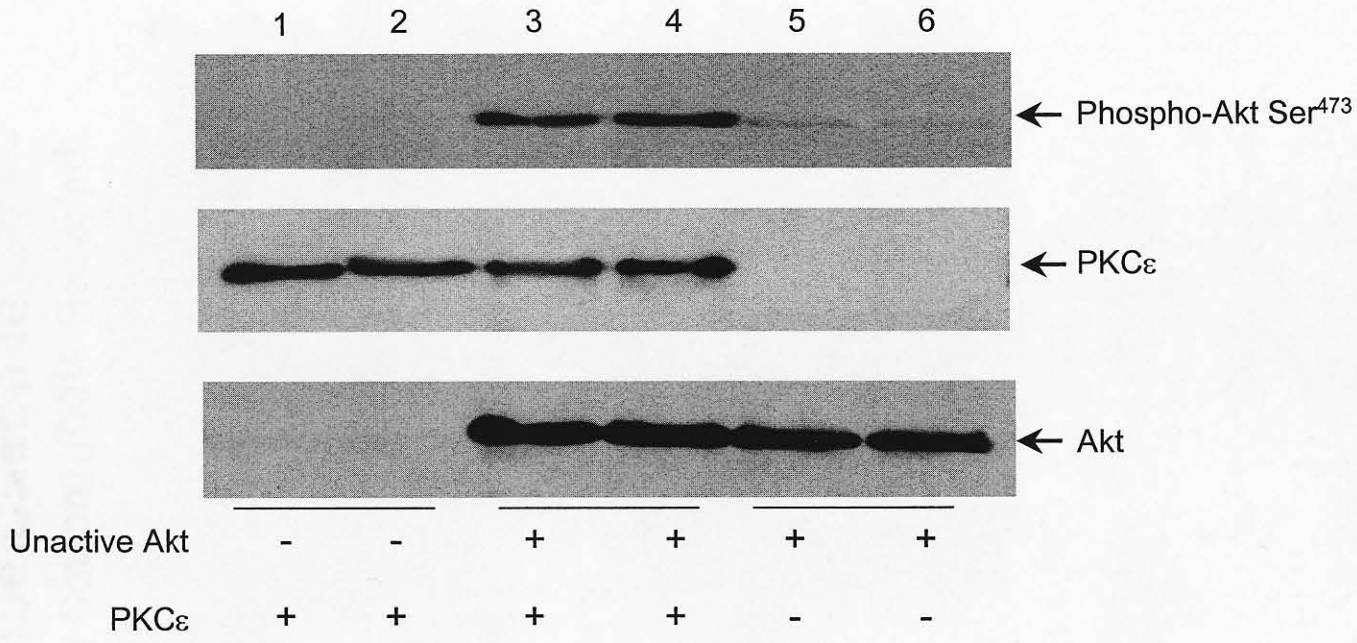
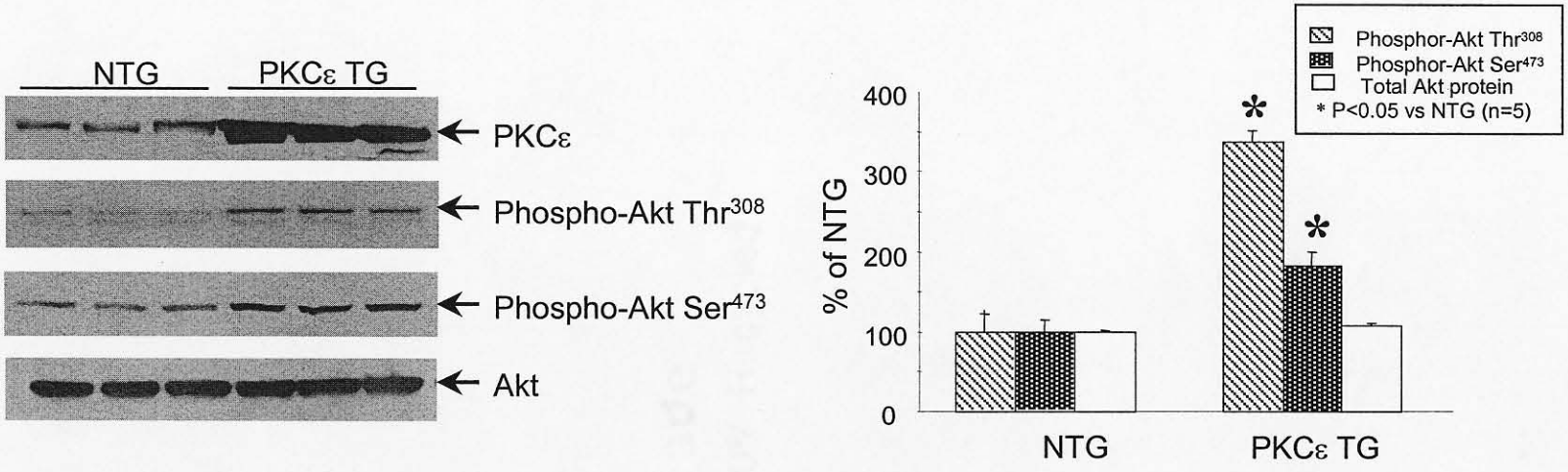

1
2
3
4
5
6
Phospho-Akt Ser473
PKCε
Akt
Unactive Akt - - + + + +
PKCε + + + + - -

Figure 22. Effect of PKCε on the phosphorylation and activation of Akt *in vivo*

a. Non-transgenic and PKCε transgenic mouse hearts were homogenized, and separated with SDS-PAGE followed by immunoblotting with Akt, PKCε and site-specific phospho-Akt antibodies. Compared with non-transgenic animals, phosphorylation of Akt on its activation sites Thr-308 and Ser-473 was significantly increased in cardioprotective PKCε transgenic mice; b. Total Akt activity and PKCε-associated Akt activity was measured by its ability to phosphorylate GSK-3β. Total cardiac cellular Akt activity was increased in PKCε cardioprotected mice when compared with non-transgenic mice. Moreover, PKCε-associated Akt activity had greater elevation when compared with the increase in the total lysate pool.

a.


NTG
PKCε TG
PKCε
Phospho-Akt Thr308
Phospho-Akt Ser473
Akt
Phosphor-Akt Thr308
Phosphor-Akt Ser473
Total Akt protein
* P<0.05 vs NTG (n=5)
% of NTG
400
300
200
100
0
*
*
NTG
PKCε TG

b.

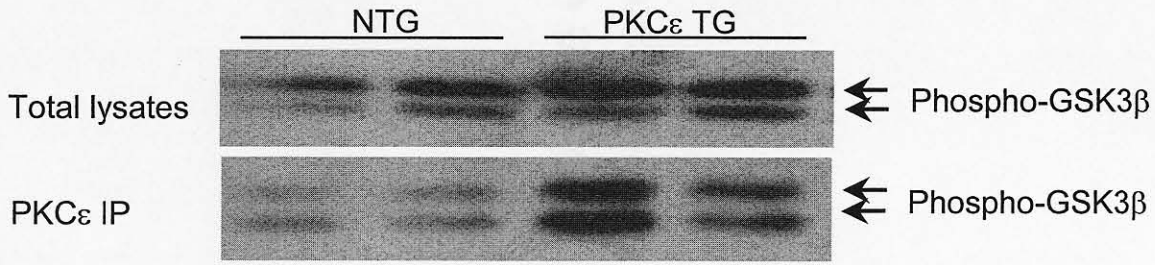
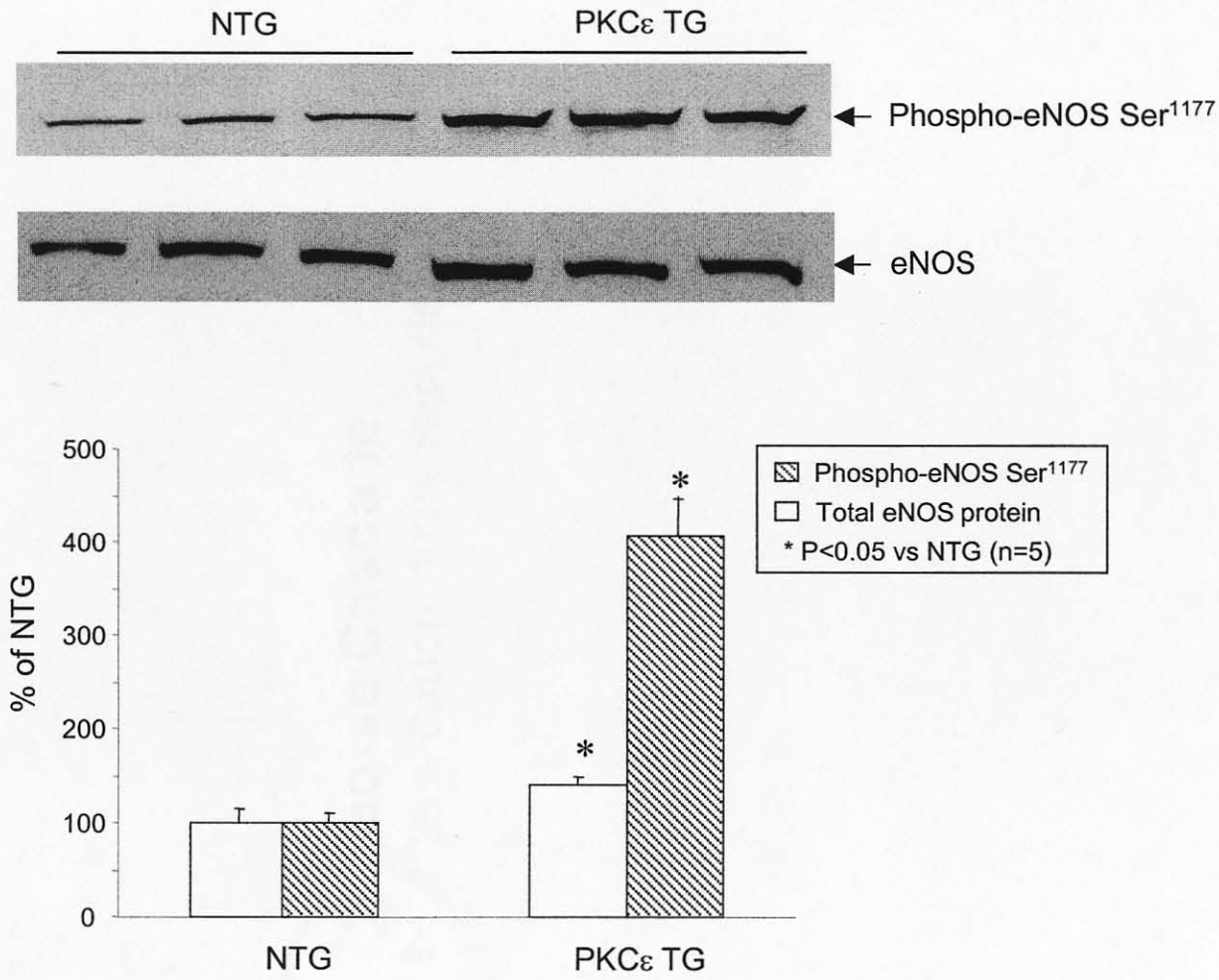

NTG
PKCε TG
Total lysates
PKCε IP
Phospho-GSK3β
Phospho-GSK3β

Figure 23. Effect of PKCε on the phosphorylation of eNOS *in vivo*. Non-transgenic and PKCε transgenic mouse hearts were homogenized and separated with SDS-PAGE followed by immunoblotting with the eNOS and site-specific phospho-eNOS Ser^{1177} antibodies. Compared with non-transgenic animals, phosphorylation of eNOS on serine-1177, the Akt-specific activation site, was also enhanced in the PKCε transgenic mice.


NTG
PKCε TG
Phospho-eNOS Ser1177
eNOS
500
400
300
200
100
0
% of NTG
Phospho-eNOS Ser1177
Total eNOS protein
* P<0.05 vs NTG (n=5)
*
*
NTG
PKCε TG

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EDUCATION

08/2001-Present: Doctoral student, Department of Physiology/Biophysics, University of Louisville. U.S.A. Major: Physiology

08/1991 - 07/1994: Master of Science, Institute of Microbiology, Chinese Academy of Sciences. P.R.China. Major: Biochemistry.

09/1987 - 07/1991: Bachelor of Science, Dept. of Microbiology, Shandong University, P.R.China. Major: Microbiology.

EMPLOYMENT AND WORKING EXPERIENCE

01/1996 – 12/2002: Senior Research Associate
Dept. of Physiology and Biophysics / Division of Cardiology, School of Medicine, University of Louisville. U.S.A.

08/1994 - 12/1995: Research Associate
Dept. of Molecular Biology, Institute of Microbiology, Chinese Academy of Sciences. P.R.China.

HONORS AND AWARDS

1. 2001: Trainee Abstract Award: American Heart Association, Scientific Sessions, Anaheim, CA.

2. 1993: Third Prize in Young Scientific Research Presentation of Chinese Academy of Sciences, P.R.China.

SOCIETY MEMBERSHIP

Member, International Society for Heart Research.
Fellow, American Heart Association, Basic Sciences Council

PUBLICATIONS

1. **Zhang J**, Baines CP, Vondriska TM, Cardwell EM, Zong C, Wang GW, Ping P. Characterization of myocardial PKCε-Akt-eNOS signaling modules via functional proteomic analysis. (in preparation)

2. **Zhang J**, Baines CP, Vondriska TM, Zong C, Cardwell EM, Wang GW, Loo R, Ping P. Functional proteomic mapping of the myocardial PKCε signaling subproteome in the mouse heart: a subcellular compartment study. (Submitted to *Proteomics*)

3. **Zhang J**, Ping P, Vondriska TM, Tang XL, Wang GW, Cardwell EM, Bolli R. Cardioprotection involves activation of NF-κB via PKC-dependent tyrosine and serine phosphorylation of IκB-α. ***Am J Physiol***. 2003; 285:H1753-1758.

4. Baines CP. Song CX, Zheng YT, Wang GW, **Zhang J**, Wang OL, Guo Y, Bolli R, Cardwell EM, Ping P. Protein kinase C epsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. ***Circ Res***. 2003; 92(8):873-880.

5. Edmondson RD, Vondrika TM, Biedermant KJ, **Zhang J**, Jones RC, Zheng YT, Allen DL, Xiu JX, Cardwell EM, Pisano MR, Ping P. Protein kinase C ε signaling complexes include metabolism-and transcription/translation-related proteins. ***Mol Cell Proteomics***. 2002; 1:421-433.

6. Baines CP, **Zhang J**, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R, Ping P. Mitochondrial PKC epsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKC epsilon-MAPK interactions and differential MAPK activation in PKC epsilon-induced cardioprotection. ***Circ Res.*** 2002; 90:390-397.

7. Ping P, Song C, **Zhang J**, Guo Y, Cao X, Li RC, Wu W, Vondriska TM, Pass JM, Tang XL, Pierce WM, Bolli R. Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. ***J Clin Invest.*** 2002; 109:499-507.

8. Balafanova Z, Bolli R, **Zhang J**, Zheng Y, Pass JM, Bhatnagar A, Tang XL, Wang O, Cardwell E, Ping P. Nitric oxide induces nitration of PKC epsilon, facilitating PKC epsilon translocation via enhanced PKC epsilon-RACK2 interactions: A novel mechanism of NO-triggered activation of PKC epsilon. ***J Biol Chem.*** 2002; 277:15021-15027.

9. Song C, Vondriska TM, Wang GW, Klein JB, Cao X, **Zhang J**, Kang YJ, D'Souza S, Ping P. Molecular conformation dictates signaling module formation: example of PKC epsilon and Src tyrosine kinase. ***Am J Physiol***. 2002; 282:H1166-1171.

10. Pass JM, Gao J, Jones WK, Wead WB, Wu X, **Zhang J**, Baines CP, Bolli R, Zheng YT, Joshua IG, Ping P. Enhanced PKC beta II translocation and PKC beta II-RACK1 interactions in PKC epsilon-induced heart failure: a role for RACK1 ***Am J Physiol***. 2001; 281:H2500-2510.

11. **Zhang J**, Vondriska TM, Song C, Tang XL, Cao X, Baines CP, Pass JM, Wang S, Bolli R, Ping P. Protein kinase C epsilon-Src modules direct signal transduction in nitric oxide-induced cardioprotection: complex formation as a means for cardioprotective signaling. ***Circ Res.*** 2001; 88:1306-1313.

12. Pass JM, Zheng Y, Wead WB, **Zhang J**, Li RC, Bolli R, Ping P. PKC epsilon activation induces dichotomous cardiac phenotypes and modulates PKC epsilon-RACK interactions and RACK expression. ***Am J Physiol***. 2001; 280:H946-955.

13. Ping P, **Zhang J**, Pierce WM Jr, Bolli R. Functional proteomic analysis of protein kinase C epsilon signaling complexes in the normal heart and during cardioprotection. ***Circ Res.*** 2001; 88:59-62.

14. Li RCX, Ping P, **Zhang J**, Wead WB, Cao X, Gao J, Zheng Y, Huang S, Han J, Bolli R. PKC epsilon modulates NF-kappa B and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. ***Am J Physiol***. 2000; 279:H1679-1689.

15. Ping P, **Zhang J**, Huang S, Cao X, Tang XL, Li RC, Zheng YT, Qiu Y, Clerk A, Sugden P, Han J, Bolli R. PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. ***Am J Physiol***. 1999; 277:H1771-1785.

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18. Ping P, Takano H, **Zhang J**, Tang XL, Qiu Y, Li RCX, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. ***Circ Res.*** 1999; 84:587-604.

19. Bolli R, Dawn B, Tang XL, Qiu Y, Ping P, Xuan YT, Jones WK, Takano H, Guo Y, **Zhang J**. The nitric oxide hypothesis of late preconditioning. ***Basic Res Cardiol***. 1998; 93:325-338.

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22. **Zhang J**, Xu JL. Oleyl oleate synthesis by immobilized lipase from *Candida sp.-1619*. ***Chinese J of Biotechnology***.1995; 11:243-252.

23. Xu JL, **Zhang J**. Studies on the lipase from *Penicillium Camembertii-PG3.* ***Acta Mycologica Sinica***. 1995; 14:136-142.

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SELECTED ABSTRACTS

1. **Zhang J**, Zong C, Vondriska TM, Qiao X, Cardwell EM, Ping P. Proteomic mapping of cardiac protective signaling complexes using blue native electrophoresis and bioinformatic modeling. ***FASEB J***. 2003; 17:A76.

2. Vondriska TM, Zhang S, **Zhang J**, Qiao X, Zheng YT, Cardwell EM, Ping P. Oracle regulates cardiac PKCε signaling complexes in preconditioning. ***FASEB J***. 2003; 17:A106.

3. Zhang S, Baines CP, **Zhang J**, Vondriska TM, Qiao X, Guo Y, Bhatnagar A, Bolli R, Ping P, Wang GW. Acrolein exacerbates ischemic injury in the heart and disrupts PKCε protective signal transduction. ***FASEB J***. 2003; 17:A880.

4. **Zhang J**, Vondriska TM, Wang GW, Baines CP, Stidam JM, Cardwell EM, Ping P. Characterization of a signaling complex by its protein composition, molecular architecture, and spatial distribution: defining the role of PKCε-Akt-eNOS modules in cardioprotection via functional proteomics. ***Circulation***. 2002; 106:II-6.

5. Ping P, **Zhang J**, Vondriska TM, Guo Y, Stidam JM, Cardwell EM, Wang GW, Bolli R. Cardiac-specific transgenesis of iNOS confers an infarct-sparing effect: signaling mechanisms involve site-specific iNOS tyrosine phosphorylation by Src tyrosine kinase. ***Circulation***. 2002; 106: II-56.

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20. Song CX, Cao X, **Zhang J**, Vondriska TM, Bolli R, Ping P. PKCε binds to and activated Src tyrosine kinase in *vitro*: Characterizing a cardiac PKCε-Src module. ***J Mol Cell Cardiol*** (suppl.), 2001.

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30. Gao JM, Zheng YT, **Zhang J**, Bolli R, Li CX, Wead WB, Ping P. Transgenic overexpression of the η isoform of PKC does not trigger cardiac hypertrophy. ***J Mol Cell Cardiol***. 2000; 32: A58.

31. **Zhang J**, Bolli R, Tang XL, Jones WK, Ping P. Direct demonstration of both tyrosine and serine phosphorylation of IκB during ischemic preconditioning in conscious rabbits. ***Circulation*** 1999, 100: I-56.

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34. Li CX, Ping P, **Zhang J**, Cao X, Zheng YT, Gao JM, Xuan YT, Wead WB, Joshua IG, Bolli R. PKCε activates NFκB and AP-1 via a ERK-dependent signaling pathway in the adult rabbit cardiac myocytes. ***J Mol Cell Cardiol***. 1999, 31: A18.

35. **Zhang J**, Wead WB, Jones WK, Wu X, Gao JM, Kong DY, Li CX, Zheng YT, Ping P. Activation of PKCε induces hypertrophy and heart failure in a dose-dependent fashion in mice. ***J Mol Cell Cardiol***. 1999, 31: A18.

36. Pass JM, **Zhang J**, Zheng YT, Li CX, Ping P. RACK expression is coordinated with PKCε expression in the mouse heart. ***J Mol Cell Cardiol***. 1999, 31: A19.

37. Ping P, Li RCX, **Zhang J**, Kong D, Jones WK, Zheng YT, Cao X, Bolli R. Identification of two distinct mechanisms for the activation of p44/p42 MAPKs in vivo by a PKC epsilon dependent signaling pathway in transgenic mice. ***Circulation*** 1998, 98:I-416.

38. Ping P, **Zhang J**, Li CX, Tang XL, Qiu Y, Banerjee S, Zheng YT, Bolli R. Ischemic preconditioning (PC) induces selective activation of Src and Lck tyrosine kinases in conscious rabbits via a PKC-dependent pathway. ***Circulation*** 1998, 98:I-71.

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40. Ping P, Kong D, Jones WK, Li CX, **Zhang J**, Cao X, Wu X, Wead WB, Harris PD, Joshua IG, Bolli R. Cardiac targeted expression of PKC epsilon isoform selectively alters PKC activity in the mouse heart. ***FASEB J***. 1998, 12:A-75.

41. Ping P, **Zhang J**, Cao X, Qiu Y, Tang XL, Clerk A, Sugden P, Bolli R. Activation of the P38MAPK and the p46/p54 JNKs after brief episodes of ischemia/reperfusion in the heart of conscious rabbits. ***J Mol Cell Cardiol***. 1998, 30: A-263.

42. Ping P, Cao X, Kong D, **Zhang J**, Li CX, Yang Y, Bolli R. PKC epsilon isoform induces activation of the p42/p44 MAPKs and the p46/p54 JNKs in adult rabbit cardiac myocytes. ***J Mol Cell Cardiol***. 1998; 30:A-263.

43. Ping P, **Zhang J**, Qiu Y, Tang XL, Manchikalapudi S, Bolli R. Repetitive episodes of myocardial ischemia and reperfusion induce translocation of protein kinase C epsilon isoform in conscious rabbits, which is associated with late preconditioning against myocardial stunning. ***Circulation*** 1996; 94:I-660.

44. Xu JL, **Zhang J.** Lipase catalyzed synthesis of 2-ethyl hexanol fatty acid esters. Oral presentation, the fourth China-Japan Joint symposium on Enzyme Engineering, Oct., 1996.

45. **Zhang J,** Jia-Li Xu. Hydrolysis and Synthesis of Oleyl Oleate Catalyzed by Lipases. Poster presentation, the Second National Conference on Enzyme Engineering, ***Acta Scientiarum Naturalium Universitatis Jilinensis*** 1993, P4-6.

46. DD Gu, J.I. Xu, **Zhang J**. Immobilization of *Candida sp.-1619* Lipase for Synthesis wax-esters. Poster presentation, the Second National Conference on Enzyme Engineering, ***Acta Scientiarum Naturalium Universitatis Jilinensis*** 1993, P4-7.

BOOK CHAPTERS:

1. Pass PM, **Zhang J**, Vondriska TM, Ping P. (Newton AC eds.) Functional proteomic analysis of the protein kinase C signaling system. *Protein kinase C protocols*, Methods in molecular biology, 233: 369-385, Humana Press, 2003.

2. Vondriska TM, **Zhang J**, Ping P. (Van Eyk JE and Dunn MJ, eds.) Proteomic characterization of protein kinase C signaling tasks in the hearts. *Proteomic and genomic analysis of cardiovascular disease.* Wiley-VCH, 2003.

3. Baines CP, **Zhang J**, Ping P. Characterization of mitochondrial signaling complexes. Ping P, Hanash S, eds. *Functional proteomics: principles and practical applications*. The Humana Press Inc. (in press).

4. Rokosh G and **Zhang J** Co-editors. *Cardiac genes expression and regulation*. Methods in molecular biology, Humana Press. (in preparation)

NATIONAL MEETING ORAL PRESENTATION

Oral presentation: American Heart Association 75th Scientific Sessions, Chicago, IL. Title: Characterization of a signaling complex by its protein composition, molecular architecture, and spatial distribution: defining the role of PKCε-Akt-eNOS modules in cardioprotection via functional proteomics. November 11, 2002.